MOLECULAR WEIGHT OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES AND ITS EFFECT ON CONDENSED TANNIN BIOLOGICAL ACTIVITY

A Dissertation

by

HARLEY D. NAUMANN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Luis Orlindo Tedeschi
Co-Chair of Committee,	James Pierre Muir
Committee Members,	Barry Don Lambert
	Merwyn Mort Kothmann
Head of Department,	David Baltensperger

August 2013

Major Subject: Agronomy

ABSTRACT

Condensed tannins (CT) are polyphenolic compounds that have demonstrated biological activities in ruminants including suppression of enteric methane (CH_4) production, protein binding and suppression of gastrointestinal nematode (GIN) infections. Some forage CT have been reported to be biologically active, whereas others have demonstrated no biological activity at all. While the chemical structure of CT has been postulated to be a key contributing factor affecting biological activity, the specific factors that determine whether or not CT from a specific forage have bioactive properties remain unknown. Results from previous studies have shown that as molecular weight of CT increases, CT biological activity also increases. Others have reported no effect of CT molecular weight on biological activity. The relationship between molecular weight of CT and CT biological activity remains inconclusive. The effect of molecular weight of CT from a variety of warm-season perennial legumes commonly consumed by ruminants on biological activity has not been adequately explored. The objectives of this study were to determine if molecular weight of CT from warm-season perennial legumes could predict the biological activity of CT relative to suppression of enteric CH_4 production, protein-binding ability (PB) and anthelmintic activity, and to compare the biological activity of CT from native warm-season perennial legumes to that of the introduced species *Lespedeza cuneata,* a plant that has gained attention in recent years due its anthelmintic properties.

All or a combination of the following warm-season perennial legume species were

evaluated for *in vitro* gas production, protein-precipitable phenolics (PPP) and PB, and percent larval migration inhibition (LMI). Eight North American native warm-season perennial legumes: *Leucaena retusa* Benth. (littleleaf leadtree), *Desmanthus illinoensis* (Michx.) MacMill. Ex B.L. Rob. & Fernald (Illinois bundleflower), *Lespedeza stuevei* Nutt. (tall lespedeza), *Mimosa strigillosa* Torr. & A. Gray (powderpuff), *Neptunia lutea* (Leavenworth) Benth. (yellow puff), two ecotypes of *Acacia angustissima* var. *hirta* (Nutt.) B.L. Rob (prairie acacia), *Desmodium paniculatum* (L.) DC. var. *paniculatum* (panicledleaf ticktrefoil), and two introduced legumes: *Arachis glabrata* Benth. (rhizoma peanut) and *Lespedeza cuneata* (Dum. Cours.) G. Don (sericea lespedeza) were included.

In vitro CH_4 production regressed on CT M_W resulted in a R^2 of 0.0009 ($P = 0.80$). There was no correlation between PPP or PB and M_W of CT (R^2 0.11; $P = 0.17$ and R^2 0.02; $P = 0.54$, respectively). There was a weak correlation between CT M_W and percent LMI (R^2 0.34; $P = 0.05$). The results of our study strongly suggested that CT M_W does not explain the biological activities of enteric methane suppression or protein-binding ability. Condensed tannin M_W may be involved in anthelmintic activity of CT from the forage legumes surveyed. North American native legumes containing biologically active CT, as compared to introduced species, were identified as having promise for use in ruminant diets.

ACKNOWLEDGEMENTS

First, I would like to give thanks to my committee, Dr. Luis Tedeschi, Dr. Jim Muir, Dr. Barry Lambert and Dr. Mort Kothmann, for their mentorship, friendship and for all they have contributed throughout the course of my doctoral program. Each set high expectations, as well as offered a unique perspective that enabled me to get the most out of this experience. There are many thanks that I could give to each of them for all they have done, including the extensive reviews of the work presented in this dissertation, but I would like to mention those that I believe to be most memorable. Specifically, I thank Dr. Luis Tedeschi, for challenging me to accomplish more than I had originally planned or thought was possible. I thank Dr. Jim Muir for his patience and leadership in improving my ability to write scientifically and in proper English (though the use of Texan probably predominates throughout these acknowledgements). To Dr. Barry Lambert, thanks for being a great mentor and thought partner as I worked through the trials and tribulations associated with graduate research. To Dr. Kothmann, thank you not only for your mentorship in the classroom, but for also providing opportunities to learn in the field as well.

I also owe many thanks to Dr. Ann Hagerman. She welcomed me into her lab as a complete stranger and provided me with the tools necessary to complete this body of work. Though not a member of my committee, she contributed to my knowledge and development as though she was.

I would like to thank my parents, Claudia and Richard, for their great generosity and willingness to help out. Their sacrifice, especially during times of my absence, was greatly appreciated. And thanks to my brother Rhett, for all of his support and encouragement as I transitioned from a mind-numbing corporate career to a more meaningful and intellectually stimulating lifework.

Finally, and without a doubt most importantly, thanks to my wife and daughter, Lindsey and Olive, for their immeasurable patience and support while I achieved one of my life goals. Had it not been for them, this effort would not have been possible. And because of them, this achievement is considerably more meaningful.

NOMENCLATURE

CT	Condensed tannins
ECT	Extractable condensed tannins
PBCT	Protein-bound condensed tannins
FBCT	Fiber-bound condensed tannins
M_W	Relative weight-average molecular weight
PPP	Protein-precipitable phenolics
PB	Protein bound
LMI	Larval migration inhibition
GIN	Gastrointestinal nematode
HC	*Haemonchus contortus*

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

NOMENCLATURE vi

TABLE OF CONTENTS vii

LIST OF FIGURES ix

LIST OF TABLES xi

CHAPTER I INTRODUCTION AND LITERATURE REVIEW 1

- Introduction 1
- Chemistry of Condensed Tannins 5
- Biological Activity 8
- Formation of Complexed Substances 10
- Enteric Methane Suppression 17
- Anthelmintic Activity 19
- Soil Carbon and Nitrogen Cycling 23
- Tannin Research 26
- Summary 26

CHAPTER II MOLECULAR WEIGHT AND PROTEIN-PRECIPITATING ABILITY OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES 28

- Introduction 28
- Materials and Methods 31
  - Experimental Forages 31
  - Condensed Tannin Quantification 32
  - Condensed Tannin Molecular Weight 33
  - Protein Precipitability 33
    - Crude Plant Extracts 33
    - Protein Precipitation 33
  - Statistical Analyses 35
- Results and Discussion 35
  - Conclusions 44

CHAPTER III EFFECT OF MOLECULAR WEIGHT OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES ON RUMINAL METHANE PRODUCTION *IN VITRO* 46

Introduction 46
Materials and Methods 47
Experimental Forages 47
Condensed Tannin Quantification 48
Condensed Tannin Molecular Weight 49
In vitro Fermentation 49
Statistical Analyses 52
Results and Discussion 52
Condensed Tannin Concentration and Molecular Weight 52
In vitro Gas Production 54
Volatile Fatty Acids 59
Molecular Weight of Condensed Tannins and Bioactivity 62

CHAPTER IV EFFECT OF MOLECULAR WEIGHT OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES ON *IN VITRO* LARVAL MIGRATION INHIBITION OF *HAEMONCHUS CONTORTUS* 66

Introduction 66
Materials and Methods 68
Experimental Forages 68
Condensed Tannin Quantification 68
Condensed Tannin Molecular Weight 69
In vitro Larval Migration Inhibition 69
Forage 70
Larvae 70
Fermentation and Condensed Tannin Extraction 71
Larval Migration Inhibition 71
Statistical Analyses 72
Results 73
Fractional Concentration of Condensed Tannins 73
Relative Molecular Weight of Condensed Tannins 73
In vitro Larval Migration 74
CT M_W and Concentration on LMI 74
Discussion 74
Conclusions 81

CHAPTER V SUMMARY AND CONCLUSIONS 82

LITERATURE CITED 84

LIST OF FIGURES

FIGURE Page

1.1 A hydrolysable tannin (gallotannin) composed of esters of gallic acid linked to a sugar core. 2

1.2 Basic structure of a condensed tannin showing stereochemistry, interflavan linkages and B-ring hydroxylation. 3

2.1 Effects of weight-average molecular weight (M_W) of condensed tannins from warm-season perennial legumes on: a. protein-precipitable phenolics (PPP g/kg DM); b. amount of protein bound by protein precipitable phenolics (PB g/kg DM); c. amount of protein bound per g of protein-precipitable phenolics (PPP/PB). 41

2.2 Effects of total condensed tannin concentration (% TCT) on a DM basis of warm-season perennial legumes on: a. protein-precipitable phenolics (PPP g/kg DM); b. amount of protein bound by protein precipitable phenolics (PB g/kg DM); c. amount of protein bound per g of protein-precipitable phenolics (PPP/PB). 42

3.1 *In vitro* methane production by fermentation of warm-season perennial legumes..... 56

3.2 *In vitro* total gas production by fermentation of warm-season perennial legumes..... 57

3.3 *In vitro* volatile fatty acid production by fermentation of warm-season perennial legumes: a.) acetate production, b.) propionate production, c.) butyrate production, d.) total volatile fatty acid production..... 60

3.4 Effects of a.) acetate:propionate and b.) total volatile fatty acid concentration on *in vitro* methane production by fermentation of warm-season perennial legumes; c.) Effect of total condensed tannin concentration (percent dry matter) on volatile fatty acid production by *in vitro* fermentation of warm-season perennial legumes. 61

3.5 Effects of a.) extractable condensed tannin molecular weight and b.) total condensed tannin (ECT) concentration on *in vitro* methane production by fermentation of warm-season perennial legumes. 63

4.1 Percent larval migration inhibition of L3 stage *Haemonchus contortus* by condensed tannins from warm-season perennial legumes. 77

4.2 The effect of condensed tannin molecular weight on percent larval migration inhibition of L3 stage *Haemonchus contortus.* 78

4.3 Effects of a.) extractable condensed tannin (ECT) concentration b.) protein-bound condensed tannin (PBCT) concentration c.) fiber-bound condensed tannin (FBCT) concentration and d.) total condensed tannin concentration (TCT; as a percent of dry matter) on percent larval migration inhibition of L3 stage *Haemonchus contortus.* 79

LIST OF TABLES

TABLE Page

1.1 Effect of molecular weight (MW) of condensed tannins on the formation of complexed substances. 11

1.2 Effects of dietary condensed tannins on gastrointestinal parasite infections in sheep and goats..... 21

2.1 Percent condensed tannins (ECT: extractable condensed tannins; PBCT: protein-bound condensed tannins; FBCT: fiber-bound condensed tannins; TCT: total condensed tannins), condensed tannin molecular weights (M_n: number-average molecular weight; M_W: weight-average molecular weight), and condensed tannin protein precipitating ability (PPP: protein-precipitable phenolics, g/kg dry matter; PB: amount of protein bound, g/kg dry matter; PB:PPP: ratio of amount of protein bound to protein precipitable phenolics) from warm-season perennial legumes. 38

3.1 Concentrations (% DM) of condensed tannin fractions of warm-season perennial legumes. 53

3.2 Relative number average molecular weight (M_n), weight average molecular weight (Mw), polydispersity index (PDI), and approximate degree of polymerization (~DP) of condensed tannins from warm-season perennial legumes. 55

4.1 Concentrations (% DM) of condensed tannin fractions and weight-average molecular weights (M_W) of warm-season perennial legumes. 76

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

Introduction

Tannins exist in nature as either hydrolysable tannins (HT) or condensed tannins (CT). Hydrolysable tannins (Fig. 1.1) are esters of gallic acid linked to a sugar core, usually glucose (HT Type 1 or gallotannins) or esters of ellagic acid, also linked to a sugar core (Types II-IV, which include ellagitannin, dehydroellagitannin, and oxidatively transformed dehydroellagitannin) (Buzzini et al., 2008; Okuda and Ito, 2011; Tharayil et al., 2011). On the other hand, CT or proanthocyanidins are polyhydroxy-flavan-3-ol oligomers (Fig. 1.2). Condensed tannins are polyphenolic compounds synthesized by plants in the phenylpropanoid pathway. They represent one of the most abundant polyphenolic compounds, second only to lignin (Tharayil et al., 2011). Condensed tannins have been defined as astringent, high molecular weight polyphenolic compounds that characteristically bind and precipitate proteins (Feeny and Bostock, 1968; Hagerman and Butler, 1981), as well as other organic compounds such as carbohydrates and minerals (Makkar, 2003). Condensed tannins are characterized based on hdroxylation pattern, stereochemistry, functional groups and interflavan linkages

---

* Reprinted with permission from "Condensed tannins in the ruminant environment: A perspective on biological activity" by Naumann, H.D., J.P. Muir, B.D. Lambert, L.O. Tedeschi, and M.M. Kothmann, 2013. *Journal of Agricultural Sciences*, 1, 8-20,

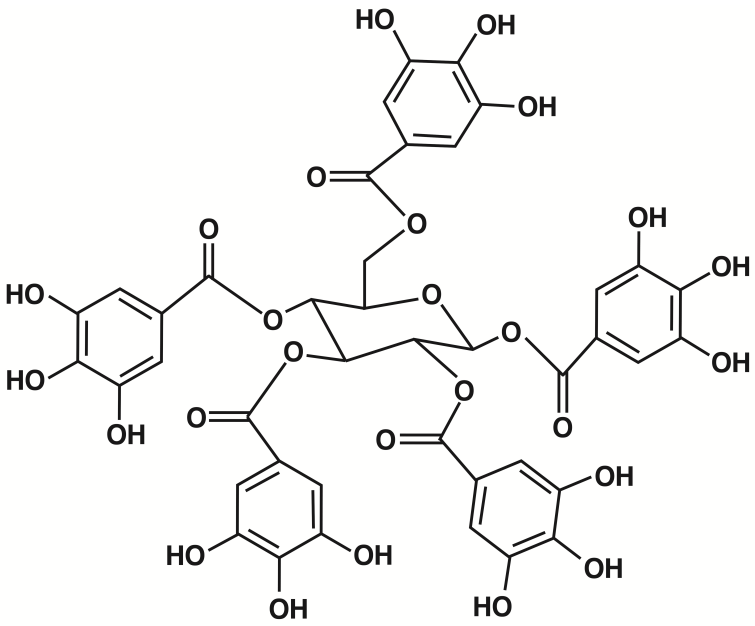

Fig. 1.1. A hydrolysable tannin (gallotannin) composed of esters of gallic acid linked to a sugar core.

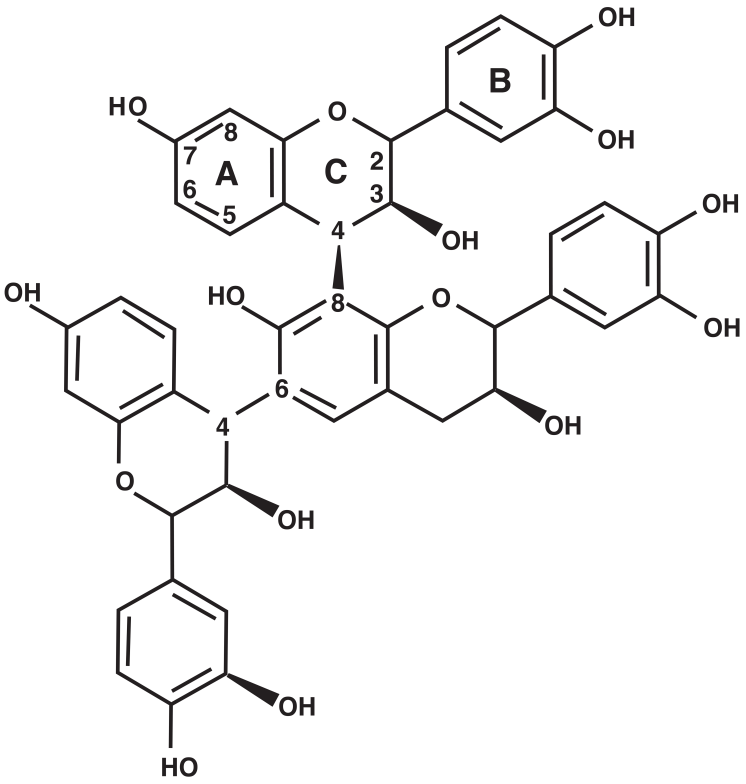

Fig. 1.2. Basic structure of a condensed tannin showing stereochemistry, interflavan linkages and B-ring hydroxylation.

(Monagas et al., 2010; Tharayil et al., 2011). Some tannins are known to possess characteristics of both HT and CT (Makkar, 2003). Hydrolysable tannins and CT are structurally different, and the first are several times referred to as potentially toxic to ruminants when large quantities are consumed (Waghorn, 2008).

The complex characteristics of CT such as variability in extent of condensation, heterogeneity of functional groups and degree of polymerization contribute to difficulty in elucidating the role and modes of action relative to biological activities of these compounds. These are constraints to understanding the extent of biological impacts of CT, not only to ruminants that consume them, but also to the ecosystem in general.

Early studies reported that, with the exception of *Lespedeza cuneata* Don, *Onobrychis viciifolia* Scop., *Trifolium arvense* L., *Lotus corniculatus* L. and *Lathyrus pratensis* L., most herbaceous species did not contain CT (Bate-Smith, 1973b). However, CT have since been isolated from the vegetative parts of many plant species (Foo and Porter, 1980; Foo et al., 1982; Williams et al., 1983; Meagher et al., 2006), and from both gymnosperms and angiosperms (Tharayil et al., 2011). It has been postulated that CT synthesis by plants is important because of their ability to provide effective defense against herbivores (Bate-Smith, 1973a). However, Beart et al. (1985) suggested that this advantage is a secondary effect in plants and that CT are not synthesized for the sole purpose of plant defense.

Concentrations of CT in plants are influenced by several factors. The infection with microorganisms, namely fungal pathogens, increases CT concentration (Dixon et al., 2012). Climatic and environmental characteristics including temperature and

precipitation (Tharayil et al., 2011), quality of photosynthetically active radiation and nutrient availability (Foo et al., 1982) can also influence CT synthesis by plants. The CT concentration in plant tissues changes seasonally or as plant tissues mature. Feeny and Bostock (1968) observed an increase in the CT concentration of leaves of deciduous *Quercus robur* L., from 0.5% in April to 5% in September. They also reported that the greatest levels of insect attack corresponded with the period of time when CT were at their lowest levels, suggesting that insects feed elsewhere when CT concentrations are high.

Chemistry of Condensed Tannins

There are many phytochemical variations within CT. The stereochemistry of CT may be either cis or trans depending on the orientation of the functional group located at the C-3 and C-4 positions relative to the B ring (Fig. 1.2). Condensed tannin monomer units possessing cis stereochemistry are named epicatechin, epigallocatechin or epiafzelechin whereas those of trans stereochemistry are called catechin, gallocatechin or afzelechin. The cis (epi) stereochemistry exists more commonly than that of trans. Foo and Porter (1980) described the structures of 38 proanthocyanidin polymers isolated from the fruits, leaves, bark and phloem of 14 families of plants. Stereochemistry consisted of predominately cis flavan-3-ol units in 34 of the 38 polymers described. Foo et al. (1982) isolated proanthocyanidin polymers from the leaves, roots and flowers of herbaceous legumes commonly used as forage plants and found all polymers to be predominately cis stereochemistry. The greatest ratio of cis:trans was observed in leaves

of *Vicia sativa* L. and was 100:0. The smallest cis:trans ratio was observed in leaves of *Robinia fertilis* Ashe and was 55:45. Meagher et al. (2006) described the structures of floral proanthocyanidins from 10 species of *Trifolium* and also found flavan-3-ol units consisting predominately of cis stereochemistry.

Condensed tannins are also characterized based on hydroxylation of the proanthocyanidin B ring, where a tri-hydroxylated B ring is a prodelphinidin (PD) unit and a di-hydroxylated B ring is a procyanidin (PC) unit (Foo and Porter, 1980; Tharayil et al., 2011). Depending on stereochemistry, a proanthocyanidin PD may also be called a gallocatechin or epigallocatechin, whereas a proanthocyanidin PC may be called a catechin or epicatechin. The ratio of PC:PD is not constant within plant species but does vary among different plant species (Jones et al., 1976), with PC occurring more commonly and in greater quantities than PD (Bate-Smith, 1975). Proanthocyanidin polymers from 28 of the 38 described by Foo and Porter (1980) were predominately PC. Characterization of the structure of CT synthesized in *Lotus* spp. by Meagher et al. (2004) confirmed the PC:PD of *L. corniculatus* was 84:16, while that of *L. pedunculatus* was 19:81.

The interflavan linkages of polyphenolic compounds (Figure 1.2) are characterized as either A-type or B-type. The A-type linkage is C4-C8 with additional ether bonds of C2-O-C5 or C7. The B-type linkages are C4-C6 or C8, with C4-C8 linkages occurring more commonly (Monagas et al., 2010). Condensed tannins possessing B-type interflavan linkages occur more commonly than those possessing A-type linkages (Li et al., 2010). These differences are relevant since interflavan linkages

of polyphenolic compounds may be related to their solubility, as Bate-Smith (1975) observed that A-type PC were less soluble in water than B-type PC at ambient temperatures.

Condensation of CT is related to the degree of polymerization (DP) of monomer units of proanthocyanidins into dimers, trimers, tetramers and higher oligomers. The DP of CT varies both within and among species. Leaves of *Trifolium affine* L.*, Onobrychis viciifolia* Scop. (Jones et al., 1976), and fruits of *Diospyros kaki* L. (Li et al., 2010) contained CT with DP and MW ranges of 19-29 monomer units and 5800-8700 Da, 57-94 monomer units and 17,000-28,100 Da and 19-47 monomer units and 7,000 to 20,000 Da respectively. It has been reported that CT from leaves of *Lotus pedunculatus* have a DP ranging from 23-24 monomer units (Jones et al., 1976) up to an average DP of 44 monomer units (Meagher et al., 2004). Adding to the complexity of CT chemistry, the possible number of stereoisomers contained within the CT compound increases as the molecular weight increases beyond a DP of 4 (Monagas et al., 2010).

Condensed tannins may either be galloylated or non-galloylated. Galloylated CT are composed of flavan-3-ol subunits containing gallic acid or galloyl groups at the C-3 position, and are limited to synthesis by dicot plants (Okuda and Ito, 2011). Examples of galloylated CT are epigallocatechin gallate and epicatechin gallate, the primary tannins found in green tea. Okuda and Ito (2011) suggested that epigallocatechin gallate and epicatechin gallate are not considered CT due to a lack of polymerization. However, Li et al. (2010) observed CT from fruits of *Diospyros kaki* L. composed of multiple terminal and extender units of these galloylated CT with average DP ranging from 19-47

monomer units. Galloylation of CT could be associated with higher MW compounds. Núñez et al. (2006) observed galloylation of flavan-3-ol compounds relative to the DP in three varieties of grape seeds. As the DP of flavan-3-ols increased, the level of those that were non-galloylated decreased.

Molecular weights of CT are dependent upon hydroxylation patterns, DP, and the number and types of functional groups contained within the polyphenolic compound. While the majority of CT synthesized by plants are 500 to 20,000 Da (Mané et al., 2007), there is evidence that oligomers of several hundred thousand Da exist (Williams et al., 1983). Williams et al. (1983) reported that molecular weight of CT synthesized depends on the plant species rather than the maturity of plant tissues or seasonality, while others have suggested that CT structures and MW vary with plant tissue maturity, seasonality and climatic conditions (Goldstein and Swain, 1965; Núñez et al., 2006; Okuda and Ito, 2011).

Biological Activity

The biological activities of CT are related to one or a combination of factors including MW, DP, stereochemistry, hydroxylation and functional groups contained within the polyphenolic compound. Further, they are related to their ability to complex with proteins, lipids, carbohydrates and minerals, the basis of which are structural chemistry and pH of the environment (Smith et al., 2005). Effects of the activities of CT have been observed in ruminant animal digestion, the focus of the present review, nutrient cycling in the environment and as a nutraceutical, a component of a food that

provides health benefits related to the treatment and prevention of disease for both livestock and humans.

Condensed tannins in plant tissues exist in three fractions: protein-bound CT (PBCT), fiber-bound CT (FBCT) and extractable CT (ECT) (Terrill et al., 1992; Wolfe et al., 2008). The concentration of CT in each fraction is dependent upon several factors, including climate and nutrient induced levels of stress (Mansfield et al., 1999; Veteli et al., 2007), age and anatomical origin of plant tissue, and varies both among and within plant species. The relative abundance of these three fractions may affect the biological activity of CT.

Two commonly used assays for measuring CT in forage samples are vanillin and butanol-hydrochloric acid. The vanillin assay is based upon reaction with the flavonoid A-ring, whereas bu-HCl is based upon acid hydrolysis and the resulting formation of anthocyanidins (Mole and Waterman, 1987). Results may vary greatly between the two assays (Mole and Waterman, 1987) and may be impacted by factors including collection, storage and processing of sample material.

Sample preparation can affect end results as well (Wolfe et al., 2008). Hagerman (1988) recommended analyzing fresh tissue upon collection to minimize confounding effects associated with plant tissue preservation, and preferred freeze-drying to oven drying when fresh tissue could not be analyzed. In agreement, Terrill et al. (1990) reported that freeze-drying resulted in more accurate assays compared to fresh freezing, oven drying and sun curing methods of plant tissue preservation. Because fresh material is not always readily available and lyophilization equipment is expensive to obtain and

maintain, air-dried material may be a viable alternative for some trials. Condensed tannin studies focusing on palatability to herbivores should work with fresh samples since that is what the animals consume. However, lyophilization of plant samples should suffice when analyzing for CT fractions. Processing method, however, may not be so important in studies that focus on post-ingestion dynamics in herbivores since effects of mastication, rumination or digestion will obviously modify CT chemistry from its pre-ingestion form.

Formation of Complexed Substances

The precipitation of proteins due to CT binding has been suggested to be the primary biological activity of CT (Mané et al., 2007). Structural chemistry determines the ability of CT to bind proteins. While the influence of molecular weight of CT on this process has been explored (Table 1.1), the specific contributing factors to CT biological activity are not known. Bate-Smith (1973a) used haemanalysis to determine the protein precipitability of CT from seeds of *Persea gratissima* and demonstrated that the ability to bind and precipitate proteins increases as DP and MW increases. The range of values for protein precipitability for the CT analyzed were reported as 0.085 to 0.12 for dimers, 0.23 to 0.33 for trimers, 0.35 to 0.40 for tetramers and ~0.50 for higher oligomers. An evaluation of flavan-3-ol monomers, dimers and trimers of catechin and epicatechin conducted by Peleg et al. (1999) also demonstrated that astringency increases as DP and MW increases. Vidal et al. (2003), confirmed this observation using proanthocyanidins

Table 1.1. Effect of molecular weight (MW) of condensed tannins on the formation of complexed substances.

Source of CT	Complexed material	Complex formation	References
Persea gratissima	Protein	Increases as MW increases	Bate-Smith (1973a)
Catechin, epicatechin	Protein	Increases as MW increases	Peleg et al. (1999)
Vitus spp., Malus spp.	Protein	Increases as MW increases	Vidal et al. (2003)
Acer rubrum	Protein	Decreases as MW increases	Tharayil et al. (2011)
Leucaena spp.	Protein	Decreases as MW increases	Huang et al. (2010)
Vitus spp., Vaccinium spp., Camellia spp.	Lipids	Increases as MW increases	Delehanty et al. (2007)

from both *Vitis* and *Malus* species, and added that increased galloylation and decreased prodelphinidin content are also contributing factors.

In contrast, Tharayil et al. (2011) suggested that CT with a greater DP and MW have a decreased capacity to bind and precipitate proteins due to a decreased conformational freedom. Huang et al. (2010) compared CT of varying molecular weights from *Leucaena* spp. on *in vitro* fermentation parameters. The average molecular weight of a *Leucaena*-hybrid Bahru CT was 2737 Da, while that of *Leucaena leucocephala* measured 2872 Da. Of the two forage types, the lower molecular weight CT from *Leucaena*-hybrid demonstrated a greater protein binding affinity with bovine serum albumin (BSA). When included at an average rate of 30 mg/g DM, the lower molecular weight hybrid resulted in a decrease in both nitrogen (N) digestibility and methane production *in vitro*. These results agree with those of Tharayil et al. (2011) and suggest that factors other than molecular weight may affect the protein binding affinity of CT (Huang et al., 2010).

Conformation of the protein also affects the affinity for CT, such that globular tightly-coiled proteins have a lower affinity for CT than proteins with a more open structure, such is the case of the salivary proline-rich proteins (PRP) family (Hagerman and Butler, 1981). The greater affinity of conformationally-open PRP for CT, as compared to other proteins, could be the result of an increased accessibility of CT phenolic groups for protein carboxyl groups allowing for greater tannin-protein hydrogen bonding (Yan and Bennick, 1995; Edelmann et al., 2002; Smith et al., 2005). Canon et al. (2011) demonstrated that as many as 15 CT composed of epigallocatechin

gallate could be bound by one 6923 Da IB5 human salivary proline rich protein and suggested that this interaction involves conformational adaptability of the protein as it structurally transits through phases of unfolding and folding throughout the binding process.

Astringency is the oral sensation of drying out and/or puckering felt in the mouth resulting from the precipitation of salivary proteins by CT (Horne et al., 2002; Shimada, 2006). Condensed tannin binding salivary proteins may provide protection to herbivores against the toxic and/or antinutritional effects associated with the consumption of plant materials containing these compounds (McArthur et al., 1995; Shimada, 2006). The formation of CT-salivary protein complexes implies that CT are free to bind dietary and/or endogenous proteins avoiding the subsequent negative effects on protein digestion and nutritional uptake (Feeny and Bostock, 1968; Horne et al., 2002). Mole et al. (1990) observed the CT binding affinity relative to proline contents of salivary proteins from 14 mammals. Different animal species presented differences in the CT-binding capacities of their salivary proteins. Of the species observed, rat salivary proteins contained 31% proline and demonstrated the greatest affinity for tannins. Salivary proteins isolated from both a pig and a cow contained the greatest levels of proline (46 and 57%, respectively) but demonstrated lower levels of affinity for CT. Salivary proteins from deer contained low levels of proline (7%) but an affinity for CT similar to the one presented from salivary proteins isolated from humans, which contained a much greater concentration of proline (31%). Results of this study indicate that, besides proline, other salivary proteins may be involved in binding CT.

Condensed tannin-protein binding affinity is greatest when the pH is nearest the isoelectric point of the protein (Hagerman and Butler, 1981). Complexation of CT to protein is affected by pH of the environment (Faithfull, 1984). Condensed tannin-protein complexes observed in the digestive tracts of ruminant animals are stable at a rumen pH of 6.5 and become dissociated at pH levels less than or equal to 3.0, those commonly encountered in the abomasum and small intestine (Jones and Mangan, 1977).

In addition to protein, CT will also complex with minerals, carbohydrates and lipids. The ability of CT to bind minerals can result in biological changes resulting from iron deficiency, reduced enzymatic activity and the inhibition of heme production (Smith et al., 2005). Faithfull (1984) observed the complexation of CT with cations and determined that, as with proteins, there are different pH values associated with complexation and dissociation that depend on the cation. Magnesium, Ca, Zn, Mn, Co and Cu all bind to CT at pH levels of 3.70 or greater. Aluminum, Fe^{3+} and Fe^{2+} bind to CT at pH levels of 3.20 or less. These observations suggest that both Fe-CT complexes and Al-CT complexes could remain stable in the abomasum and small intestine of ruminant animals. The knowledge that CT bind with Fe has resulted in the use of CT as corrosion inhibitors. For example, Rahim et al. (2011) evaluated CT extracted from the bark of *Rizophora apiculata* and observed the transformation of rust to ferric-tannates.

The ability of CT to bind to lipids has also been demonstrated. Delehanty et al. (2007) used CT isolated from *Vitis* spp., *Vaccinium* spp., and *Camellia* spp. to demonstrate that CT have the ability to bind to Lipid A, the primary cell binding site of lipopolysaccharide. Condensed tannins isolated from *Vaccinium* spp. with MW of

greater than 6000 Da and an average DP of 21 demonstrated the greatest lipid binding ability.

Further biological activities of CT have been discussed with regards to their interactions with both rumen microorganisms and ruminant digestion. The ability of CT to bind to nutrients has been suggested as the basis for inhibition of microbial activity (Smith et al., 2005). Inhibition is the result of direct interaction of CT with microbial cell wall constituents and the indirect interaction of CT with nutrients, rendering them unavailable to microorganisms (Smith et al., 2005; Patra and Saxena, 2011). Some microorganisms have demonstrated an ability to adapt to direct interaction with CT. O'Donovan and Brooker (2001) reported that the amount of protective extracellular polysaccharide produced by *Streptococcus gallolyticus* increased in response to contact with increasing levels of CT, allowing microbial growth to continue. In the same study, *Streptococcus bovis* was not effective at adapting to CT.

Protein that is bound by CT in the rumen and later dissociated in the abomasum could increase protein digestibility in the small intestine. An increase in dietary protein in the small intestine has been linked to overall improvements in animal performance, including body weight gain, wool and milk production, reproductive performance and the ability to cope with gastrointestinal nematode (GIN) burdens (Patra and Saxena, 2010).

Condensed tannin-protein complexation can shift the site of N metabolism and absorption. To demonstrate these effects, Perez-Maldonado and Norton (1996) fed sheep and goats diets containing the legumes *Desmodium intortum* and *Calliandra calothyrsus*

at a level of 300 g/kg dry matter (DM) in a basal diet of *Digitaria decumbens*. The *D. intortum* and *C. calothyrsus* diets contained CT in amounts of 9.5 g/kg and 22.5 g/kg DM, respectively. Sheep and goats consuming diets containing CT experienced as much as 21% more N reaching the abomasum as compared to those fed a diet free of CT. Kariuki and Norton (2008) confirmed the dissociation of proteins from CT post-ruminally in sheep using *Leucaena leucocephala*, *Leucaena pallida*, *C. calothyrsus*, *D. intortum*, *Acacia aneura*, and *Schinopsis* spp. (quebracho) CT extract. The rate of protein release varied among CT from the different plants surveyed such that CT-protein dissociation in the abomasum was inversely related to binding affinity.

Another effect of CT-protein binding on ruminant digestion is the shift in N excretion from urinary ammonia (NH_3-N) to fecal N. Perez-Maldonado and Norton (1996) demonstrated that fecal N increases 14% when sheep and goats are fed diets containing *D. intortum* and *C. calothyrsus* at a concentration of 300 g/kg DM. Dschaak et al. (2011) suggested a shift in N excretion also occurs in lactating dairy cows supplemented with quebracho CT extract at a concentration of 30 g/kg DM. Increased excretion of fecal N could be the result of decreased N absorption by ruminants consuming CT. However, Perez-Maldonado and Norton (1996) observed the opposite effect. When sheep and goats were fed diets containing CT, they absorbed 19% more N than those consuming a diet free of CT.

Enteric Methane Suppression

In the rumen, anaerobic methanogen microorganisms digest cellulose into forms usable by the animal. During this process of ruminal fermentation, enteric methane (CH_4) is produced from the disposal of metabolic hydrogen (H_2) (Newbold et al., 2005). Reducing equivalents that are not consumed during the formation of useful products such as volatile fatty acids could be transformed into CH_4 (Newbold et al., 2005), representing a loss of as much as 15% gross energy to the animal (Patra and Saxena 2010). Condensed tannins reduce CH_4 production by ruminants (Tedeschi et al., 2011) both *in vitro* (Huang et al., 2011; Pellikaan et al., 2011) and *in vivo* (Animut et al., 2008; Kongvongxay et al., 2011; Puchala et al., 2012).

The mechanism of action of CT on methanogenesis is not completely understood. It has been suggested that, depending on type and dose, CT may directly inhibit the growth of methanogen microorganisms in the rumen (Patra and Saxena, 2010; Williams et al., 2011). Indirect inhibition of methanogens could occur by decreasing the availability of nutrients to microorganisms in the rumen. Inhibition of methanogenesis by CT may also result in decreasing the acetate to propionate ratio, resulting from an increased transfer of hydrogen to propionate (Dschaak et al., 2011). Another possibility is that CT are hydrogen acceptors and reduce the amount of hydrogen available in the rumen to form CH_4.

In an extensive review of the effect of plant secondary metabolites on methanogenesis, Patra and Saxena (2010) compared the effects of CT on CH_4 production and fermentation in the rumen. Their review provided a basis for potentially

understanding which forage types are likely to provide a reduction in methane emission when fed to livestock. In a study assessing methane emission by goats consuming CT from different forage, Animut et al. (2008) fed *Lespedeza striata*, a combination of *Lespedeza striata* and quebracho tannin, *Lespedeza cuneata* (sericea lespedeza) and a combination of *Lespedeza striata* and sericea lespedeza. The concentrations of CT fed in these experimental diets were 151, 198, 140, and 146 g/kg DM respectively. In a second phase of their trial, the same experimental diets were fed; however, polyethylene glycol (PEG), which binds to and neutralizes CT, was added. Both DM intake and N digestibility were less across all experimental diets fed without PEG as compared with those fed with PEG. An increase in CH_4 emission in the presence of PEG (19.1 l/day) compared to diets fed without PEG (9.01 l/day) was reported, suggesting that CT from these forages may result in a reduction in CH_4 production when fed to goats. Pellikaan et al. (2011) measured *in vitro* CH_4 production with the addition of 100 g/kg CT to rumen liquid obtained from lactating dairy cows. Condensed tannins from *Schinopsis* and *Vitis* spp. decreased the amount of CH_4 produced. Kongvongxay et al. (2011) fed *Mimosa pigra*, a plant containing CT in concentrations ranging from 40 to 80 g/kg DM, to goats at rates of 25, 50, and 75% of the diet. Reduced emissions of CH_4 were observed at all levels with the greatest reduction observed at 50%.

Williams et al. (1983) observed the effects of legume forages *Astragalus cicer*, *Onobrychis viciifolia*, two cultivars of *Lotus corniculatus* and *Medicago sativa* on CH_4 production *in vitro*. Condensed tannin concentrations for these forages were 4.49, 48.5, 7.68, 9.90 and 5.17 g/kg DM, respectively. Methane production was greatest for *M.*

sativa and lower for all other forages analyzed. Although *M. sativa* contained 15% more CT than *A. cicer*, it resulted in greater CH_4 production, suggesting that factors other than CT concentration may affect CH_4 production. Molecular weights of CT were not reported in this study, but could have been a factor in CH_4 production. Huang et al. (2011) compared *in vitro* CH_4 production from cattle fed *Leucaena* spp. with CT of various MW. Molecular weights of five fractions of CT ranged from 442 to 1566 Da. The greatest MW CT resulted in the smallest amounts of both total gas and CH_4 produced, suggesting that overall organic matter digestion was reduced and that the type of CT present indirectly affected methanogenic microbial populations in the rumen.

Condensed tannins from different plant sources may affect enteric CH_4 production in different ways. Varying the concentrations of CT will also affect the amount of enteric CH_4 produced, but the greatest concentrations of CT will not always result in the greatest reductions of enteric CH_4. The reductions of enteric CH_4 production observed when feeding forages containing CT may occur at the expense of antinutritional factors such as decreased DM intake, as well as N and DM digestibility.

Anthelmintic Activity

Much of the recent work involving the feeding of forages containing CT to ruminants has been conducted to better understand their anthelmintic properties. The development of anthelmintic resistance by GIN parasites in goats has been reported as widespread (Prichard, 1990). Feeding forages containing CT to goats as helminth control may be an alternative to commercially manufactured anthelmintics (Kahiya et al., 2003;

Max et al., 2007) or as part of an integrated system to reduce future occurrences of anthelmintic resistance to synthetic commercial products. While the anthelmintic mode of action of CT on GIN is not fully understood, in a study using commercial preparations of quebracho and wattle (*Acacia saligna*) CT, Max et al. (2005) reported ingestion of excessive CT by adult nematodes, resulting in death.

When sheep and goats consume some forages containing CT, GIN parasites are suppressed (Table 1.2). However, the consumption of other forages containing CT results in no anthelmintic affect. The cause of these varying responses is unknown. Many forages containing CT have demonstrated anthelmintic activity in ruminants with GIN infections. Kahiya et al. (2003) fed mixed diets containing either 40% *Acacia karoo* or *Acacia nilotica* and reported CT concentrations to be 2.22 and 0.17 absorbance units at 550 nm (A550), respectively. Goats fed a diet containing *A. karoo* experienced reduced fecal egg counts (FEC); whereas goats fed a diet containing *A. nilotica* experienced no decrease. The lack of anthelmintic effect on goats fed the diet containing *A. nilotica* could be explained by the low concentration of CT in the diet. Brunet et al. (2008) fed fresh leaves of *Lysiloma latisiliquum* containing 9.02 g CT/kg DM to goats *ad libitum* and reported a reduction in larval establishment of GIN. The protein precipitation activity (PPA) of CT from *L. latisiliquum* measured 4.09 PPA cm^2/g DM relative to a standard (resorcinol). The control used in this study, *Brosimum alicastrum,* had a biological activity of 1.72 PPA cm^2/g DM. These results suggest that protein-precipitating activity of CT may be a factor influencing whether or not CT from specific forage possesses anthelmintic properties.

Table 1.2 Effects of dietary condensed tannins on gastrointestinal parasite infections in sheep and goats.

Plant material	Dietary CT	Animal species	Anthelmintic effects	References
Acacia karoo	Not reported	Goats	Reduced fecal egg counts	Kahiya et al. (2003)
Acacia nilotica	Not reported	Goats	No reduction in fecal egg count	Kahiya et al. (2003)
Lysiloma latisiliquum	9.02 g/kg	Goats	Reduced larval establishment	Brunet et al. (2008)
Lespedeza cuneata	24.5 g/kg	Goats	No reduction in fecal egg count	Terrill et al. (2009)
Lespedeza cuneata	49 g/kg	Goats	Reduced fecal egg count 84.6%	Terrill et al. (2009)
Lespedeza cuneata	73 g/kg	Goats	Reduced fecal egg count 91.9%; Reduced larval establishment and adult nematodes	Terrill et al. (2009)
Lespedeza cuneata	168 g/kg	Goats	Reduced fecal egg counts 88%; Reduced L3 larvae and adult nematodes	Shaik et al. (2006)
Acacia molissima	150 g/kg (drenched)	Sheep	Reduced fecal egg counts and adult nematodes	Minho et al. (2008)
Manihot esculenta	40 g/kg	Sheep	Reduced fecal egg count 41%; Reduced L3 establishment	Marie-Magdeleine et al. (2010)
Acacia polyacantha	324 g/kg	Goats	No reduction in fecal egg count	Max et al. (2007)
Hedysarum coronarium	45 g/kg	Goats	No reduction in fecal egg count	Pomroy and Adlington (2006)
Sorghum spp.	43.7 g/kg	Goats	No reduction in fecal egg count	Whitley et al. (2009)
Sorghum spp.	63.7 g/kg	Goats	No reduction in fecal egg count	Whitley et al. (2009)
Sorghum spp.	121 g/kg	Goats	No reduction in fecal egg count	Whitley et al. (2009)

Terrill et al. (2009) fed *Lespedeza cuneata* (sericea lespedeza) hay at levels of 25%, 50%, and 75% of experimental diets containing 24.5, 49, and 73 g CT/kg DM, respectively. There was no reduction in FEC reported at the 25% level. However, at both 50% and 75% levels, decreases in FEC of 84.6% and 91.9%, respectively, were observed. In addition to decreased FEC, feeding sericea lespedeza at 75% of the diet resulted in decreases in L3 larvae establishment and adult GIN. In another study observing natural deworming, Shaik et al. (2006) fed sericea lespedeza hay containing 224 g CT/kg DM to Boer goats at 75% of the diet. Reductions in FEC of 88%, as well as significant reductions in L3 larvae and adult GIN, were observed.

Minho et al. (2008) drenched sheep with CT extracts from *Acacia molissima* containing 150 g CT/kg DM and observed reductions in both FEC and adult *Haemonchus contortus* in the abomasum. However, there were no observed reductions in adult *Trichostrongylus colubriformis* in the small intestine. Marie-Magdeleine et al. (2010) fed wilted leaves from *Manihot esculenta* containing 40 g CT/kg DM to sheep and observed a 41% reduction in FEC and the reduced development of L3 larvae from eggs.

Not all forages or feeds containing CT demonstrate anthelmintic properties (Table 1.2). The following are examples where CT had little or no effect on GIN infections. Max et al. (2007) fed *Acacia polyacantha* containing 324 g CT/kg DM to goats and observed no reduction in FEC or GIN burden. Other forages such as *Hedysarum coronarium* and varieties of grain sorghum containing high concentrations of CT have been fed at levels comparable to or even greater than those of sericea

lespedeza and *L. latisiliquum* with no anthelmintic effects. For example, Pomroy and Adlington (2006) fed *H. coronarium* containing 45 g CT/kg DM to goats *ad libitum* and reported no effect on established GIN populations. The concentration of *H. coronarium* CT fed to goats was five times greater than that of the *L. latisiliquum* fed by Brunet et al. (2008). However, feeding *L. latisiliquum* resulted in decreases in fecal egg counts whereas no effect was observed for *H. coronarium*. The lack of anthelmintic activity of forages containing CT was also observed when Whitley et al. (2009) fed sorghum grain varieties containing 43.7, 63.7, and 121 mg CT/g DM to naturally infected goats with no effect on FEC. In some cases, the concentration of CT in sorghum was greater than that of feeding trials using sericea lespedeza; yet feeding sericea resulted in direct anthelmintic effects.

Multiple factors may explain the conflicting results of feeding forages with similar concentrations of CT to goats including differences in the chemistry of CT from different plants used and laboratory methods used to determine CT concentrations. However, molecular weight and concentration of CT fractions appear to be the most likely contributing factors.

Soil Carbon and Nitrogen Cycling

Condensed tannins from leaf litter can alter microbial activity and N cycling in the soil. Condensed tannins alter the N cycle and subsequently reduce microbial activity by binding N and preventing it from being released or by directly inhibiting microbial enzymes (Madritch et al., 2007). Soil microorganisms are less able to mineralize N when

it is bound by CT, which could result in a reduction in microbial activity. An additional consequence could be a reduced availability or slower release of mineralized N to the plant environment.

Verkaik et al. (2006) demonstrated that CT from *Agathis australis* reduced net N release resulting from reduced N mineralization. Talbot and Finzi (2008) confirmed the cause of altered N cycling to be the complexation of CT to protein. They determined that CT and HT from leaf litter of *Quercus rubra*, *Tsuga canadensis* and *Acer saccharum* will form tannin-protein complexes and that N cycling is affected by tannin concentrations. Litter from *A. saccharum, Q. rubra* and *T. canadensis* contained HT and CT at concentrations of 73.46 and 24.00, 53.94 and 5.63, and 37.40 and 0.33 mg/g DM respectively. Condensed tannins from the three litter types precipitated proteins in amounts of 3.39, 0.34 and 0.51 mg/mg tannin, respectively. Of the three, litter from *A. saccharum* contained the greatest amount of CT and precipitated the greatest amount of protein. However, *T. canadensis* contained less total CT than *Q. rubra* but precipitated a greater amount of protein suggesting factors other than concentration are involved in the biological activity of these CT.

A consequence of decreased N availability in soils resulting from reduced mineralization related to foliar CT could be that plants must compensate for the lack of N in their environment. Fischer et al. (2006) observed that, in an effort to meet N requirements, plants increase fine root production as foliar CT concentrations increase in an effort to meet the plant's requirements for N.

Soil carbon cycling is altered in the presence of CT as a result of altered microbial respiration (Talbot and Finzi, 2008), which affects the amount of carbon dioxide (CO_2) released to the atmosphere. The presence of foliar CT in soils can affect microbial activity differently depending on time (Madritch et al., 2007). Madritch et al. (2007) used CT obtained from *Populus tremuloides* to determine effects of CT on soil respiration. When CT was added to the soil there was an initial increase in respiration followed by a long-term negative effect of CT on respiration.

An important consequence of CT altering C and N cycling in the environment is the altering of positive and negative feedbacks on climate change drivers such as elevated levels of CO_2 and associated elevated temperature. In turn, climatic conditions can affect the synthesis of CT. Tharayil et al. (2011) observed that leaf litter of *Acer rubrum* exposed to conditions of elevated temperature and decreased precipitation resulted in the synthesis of CT predominated by procyanidins with a lower DP than CT synthesized under ambient conditions. Condensed tannin content in leaf litter at ambient temperatures consisted predominately of prodelphinidins. Núñez et al. (2006) reported that concentration of galloylation in CT was affected by climatic conditions, indicating that MW could also be related to climatic conditions and seasonality.

As a result of long-term negative effects of CT on microbial respiration, soil would likely become a lesser source of atmospheric C when biologically active CT are present. A portion of the carbon released as a result of initial, short-term increases in microbial respiration would be used by plants, which may produce more CT, a form of stored C, when exposed to increased levels of atmospheric CO_2 (Carter et al., 1999).

Tannin Research

There are many unanswered questions and unproven hypotheses related to the biological activities of CT in the ruminant environment. A common assumption is that concentration of CT determines the biological activity of the CT. A specific question worth exploring is how are specific biological activities (anthelmintic, ruminal methane reduction, protein binding ability, and anti-microbial) of CT affected by CT of differing structural chemistry. An additional research topic is whether MW of CT is related to any of the aforementioned biological activities.

As previously discussed, there are multiple hypotheses for how plant CT might suppress CH_4 production during rumen fermentation. However, the mechanisms by which plant CT inhibit larval migration and reduce FEC are known to a lesser degree. Elucidation of the modes of action of CT relative to biological activities in the ruminant ecosystem is of great interest and should be a focus of future CT research.

Summary

The role of plant CT may differ considerably with source of plants and MW of CT (Muir, 2011). When we consider the wide variety of plants consumed by grazing cattle and the even more numerous species browsed by goats (Child et al., 1985), not to mention wild ungulates, the reality of just how little we know about the role of CT on ruminant nutrition becomes evident. Interest in using native forages to improve nutrition in cattle, but more importantly small and native ruminants, is growing. This is particularly evident for legumes as reflected in the growing body of literature (McGraw

et al., 2004; Muir et al., 2005, 2011). The determination of MW of CT fractions from leguminous forage commonly fed to ruminants, relative to reduction of emissions of greenhouse gasses (e.g. methane) by ruminant animals, as well as the maintenance or improvement of the overall animal performance has not been adequately explored. Such knowledge could result in improved animal performance through use of CT containing plants for GIN suppression, nutrient-use efficiency in ruminants and decreased CH_4 emissions, the latter a major environmental concern. If we can classify CT based on the fraction and MW that cause effective reductions of emissions of methane by ruminant animals, while maintaining or improving the overall animal performance, we will be closer to harnessing the potential of plant species that develop biologically active CT and fostering the development of more specific and efficient commercial products. If we can evaluate new ruminant feed sources by quickly assaying CT characteristics in a laboratory without having to resort to time-consuming, expensive field trials, we can shortcut their use in ruminant production.

CHAPTER II

MOLECULAR WEIGHT AND PROTEIN-PRECIPITATING ABILITY OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES

Introduction

Incorporating rangeland legumes into ruminant production systems has the potential to increase availability of forage crude protein (CP) and metabolizable energy to the animal (Foster et al. 2007; Muir et al. 2011) and to decrease methane gas production (Tedeschi et al. 2011; Naumann et al. 2013a). These effects are generally attributed to the condensed tannins (CT) that are characteristic of warm-season perennial legumes (Wolfe et al. 2008). Despite some reports of anti-nutritional effects of forage CT on ruminants (Barry & Mcnabb 1999; McSweeney et al. 2005; Krueger et al. 2010), positive effects of dietary CT dominate the current literature (Perez-Maldonado & Norton 1996; Min et al. 2005; Gurbuz et al. 2008).

Dose-response relationships that support the hypothesis that the benefits of forage legumes to ruminants derive from CT have been demonstrated in many studies. Rumen degradability of crude protein declines as the level of forage CT in the rumen is increased (Azuhnwi et al. 2012). There is a dose-response effect of CT on methanogenesis (Tiemann et al. 2008). Terrill et al. (2009) reported a dose-response effect of *Lespedeza cuneata* CT as an anthelmintic. Although the dose-response relationship is strong within each of these studies, the variation in the effects of CT from different plant species suggests there are qualitative as well as quantitative effects.

The hypothesis that CT activity is dependent on structure as well as concentration is supported by several studies that directly compared CT from different species. Condensed tannins from *Calliandra calothyrsus* and *Flemingia macrophylla* more effectively increased bypass protein than CT from *Leucaena leucocephala* (Cortes et al. 2009). *Calliandra calothyrsus* and *F. macrophylla* had different effects on methane suppression, nitrogen and fiber digestibility (Tiemann et al. 2008). Anthelmintic activity was reported for ruminants consuming diets containing CT from *Lysiloma latisiliquum* (Brunet et al. 2008a) and *Acacia mearnsii* (Cenci et al. 2007), but not for those consuming *Sorghum* (Whitley et al. 2009) or *Hedysarum coronarium* (Pomroy & Adlington 2006). Although the data suggest that the different effects of CT from different plants result from differences in CT structure, other variables obscure the structural relationships. Test system variables such as animal condition, type of plant, other dietary factors such as crude protein, and environmental factors play a dominant role in animal studies. In order to analyze CT structure-activity relationships, a laboratory system was chosen to explore how tannin structure, specifically molecular weight, influences protein binding, as a surrogate for biological activity in ruminants.

Like other polyphenolic compounds, CT are highly reactive. Although oxidative activity of CT has been the primary focus of human health literature (Koleckar et al. 2008), in animal nutrition attention is focused on protein binding. There is indirect evidence that protein binding is the key biological activity of CT in ruminants. For example, the pH-dependence of leaf protein precipitation by CT mirrors the pH values found in the ruminant digestive system and suggests a mechanism of action for tannins

in ruminant digestion (Faithfull 1984). Condensed tannin-leaf protein complexes are stable at pH 6.5, but dissociate at pH levels less than or equal to 3.0 (Jones & Mangan 1977; Reed 1995). At rumen pH (~6.5) protein would be tannin-bound and thus indigestible. In the abomasum (pH < 3), protein would be released and available for digestion. Reports of suppressed enteric methane production accompanying decreased crude protein digestibility during consumption of CT (Tiemann et al. 2008), suggests a mechanistic relationship between protein binding and enteric methane production. Brunet et al. (2008a; 2008b) demonstrated a relationship between CT-protein binding and anthelmintic activity by using polyvinyl polypyrrolidone or polyethylene glycol, agents that inhibit CT-protein interactions, to reverse the beneficial effects of CT *in vivo*.

Condensed tannin structural diversity results from stereochemical or structural isomers, patterns of substitution and molecular size, and is determined by genetic and environmental programming (Scioneaux et al. 2011). For example, mono-, di- and tri-hydroxylation patterns constitute the different flavan-3-ol units, propelargonidin, procyanidin and prodelphinidin, respectively. The interflavan linkages may be B-type, at the C4-C6' or C4-C8' position, or may be A-type at the C2-O-C7' position (Yoshida et al. 2005). Additions of galloyl groups or sugars at the C-3 position add further structural diversity (Okuda and Ito 2011). The degree of polymerization ranges from small oligomers including dimers, trimers, and tetramers and to larger polymers of up to 20 or more subunits.

Protein precipitation is a complex function of molecular characteristics of the tannin including its polarity, molecular weight, degree of galloylation and flexibility

(Hagerman et al. 1998; Soares et al. 2007; Hagerman 2012). Any of these factors may be responsible for the differential biological activities of different tannins. One of the few studies directly relevant to animal nutrition that evaluated structural details of CT demonstrated a relationship between molecular weight of the CT and ruminal digestibility (Aerts et al. 1999). The importance of molecular weight in determining protein precipitation by tannins has been demonstrated for groups of closely related compounds (da Silva et al. 1991; Kawamoto et al. 1995; Sarni-Manchado et al. 1999). In general, larger molecular weight tannins precipitate protein more efficiently than lower molecular weight tannins. The objectives of this study were to further test the relationship between molecular weight and biological activity of CT from warm-season perennial legumes using protein precipitation as the surrogate for biological activity and using gel permeation chromatography (GPC) to assess molecular weight of the mixtures of CT extracted from these important forage legumes. Attention was given to identifying warm-season perennial legumes that produce biologically active CT and potentially increase efficiency of protein utilization by ruminants.

Materials and Methods

Experimental Forages

The plant material consisted of leaves from North American native *Leucaena retusa* Benth. (littleleaf leadtree), *Desmanthus illinoensis* (Michx.) MacMill. Ex B.L. Rob. & Fernald (Illinois bundleflower), *Lespedeza stuevei* Nutt. (tall lespedeza), *Mimosa strigillosa* Torr. & A. Gray (powderpuff), *Neptunia lutea* (Leavenworth) Benth. (yellow

puff), two ecotypes (STP5, Cross-timbers; STX, South Texas) of *Acacia angustissima* var. *hirta* (Nutt.) B.L. Rob (prairie acacia), and *Desmodium paniculatum* (L.) DC. var. *paniculatum* (panicledleaf ticktrefoil), collected in Stephenville, Texas, USA (32° 15' N, 98° 12' W, altitude 395 m). *Arachis glabrata* Benth. (rhizoma peanut), selected as a negative control due to its typical low CT concentration, and *Lespedeza cuneata* (Dum. Cours.) G. Don (sericea lespedeza) are both introduced species also collected in Stephenville. All plants were grown on a Windthorst sandy loam soil (Udic Paleustalf; 10 mg/kg nitrate-N, 13 mg/kg P, 206 mg/kg K, 1416 mg/kg Ca, 247 mg/kg Mg, 15 mg/kg S, 152 mg/kg Na, 10.12 mg/kg Fe, 0.59 mg/kg Zn, 1.10 mg/kg Mn, and 0.22 mg/kg Cu using Mehlich III extraction; Mehlich, 1984). Leaves were dried at 55°C in a forced air oven for 48 h, then ground to pass a 1-mm screen in a sheer mill (Wiley Arthur H. Thomas Co., Philadelphia, PA, USA) and stored for later chemical analysis.

Condensed Tannin Quantification

Total CT concentrations were determined for two replicated samples of each plant species as described by Terrill et al. (1992). Extractable CT (ECT) was extracted from 250 mg of plant tissue with 10 mL of a 70:30 (v/v) acetone:water followed by 10 mL of diethyl ether. Protein-bound CT (PBCT) was extracted from the residue with 10 mL of sodium dodecyl sulfate (1% w/v)-mercaptoethanol (5% v/v) containing Tris hydrochloride (0.01 M). Fiber-bound CT (FBCT) was determined using the residue after ECT and PBCT extraction. Condensed tannins in each fraction were determined based on absorbance at 550 nm following reaction with butanol-HCl (5% v/v HCl). Species-specific standards were created for each plant species analyzed (Wolfe et al. 2008) using

CT extracts purified on Sephadex LH-20 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and lyophilized to recover purified CT.

Condensed Tannin Molecular Weight

Molecular weights of CT from each species were determined by gel permeation chromatography (GPC) (Waters, Milford, MA) as described by Huang et al. (2010) using a PLgel 3 μm Mixed-E column (Agilent Technologies, Santa Clara, CA) and tetrahydrofuran as the mobile phase. Purified CT extracts were dissolved in tetrahydrofuran to a final concentration of 0.5 mg/mL and 50 μL was injected at a flow rate of 1.0 mL/min at 25°C. Relative weight-average molecular weights (M_W) were calculated based on a calibration curve developed using Polystyrene Low EasiVials (Agilent Technologies, Santa Clara, CA) comprising polystyrene standards ranging from 162 to 38,640 Da.

Protein Precipitability

The scaled-down method described by Hagerman and Butler (1978) was used to determine protein precipitability of CT in three replicate crude plant extracts.

Crude plant extracts

Crude plant extracts were prepared for each plant species by extracting 50 mg of plant tissue with 1 mL of 50:50 (v/v) methanol:water on a rotator for 15 min followed by centrifugation at 16,000 x g for 5 min at 4 °C.

Protein precipitation

To determine PPP or PB, 50 μl of supernatant from crude plant extracts were combined with 250 μL Buffer A (0.20 M acetic acid, 0.17 M NaCl, pH 4.9), 50 μL

bovine serum albumin (BSA) (5 mg/mL in Buffer A) and 50 µL 50:50 (v/v) methanol:water, and incubated at room temperature for 30 min before centrifuging at 16,000 x g for 5 min at 4°C. Supernatants were removed by vacuum aspiration and the protein-phenolic pellet was washed with 100 µL Buffer A before re-centrifuging and aspirating.

To determine PPP, the pellets were dissolved in 800 µL of sodium dodecyl sulfate (1% w/v)-triethanolamine (5% v/v) before adding 200 µL of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 M HCl). Absorbances at 510 nm were read after 15 min. Standard curves for PPP were prepared with stock solutions of CT extracts (1 mg/mL) in deionized water. Levels of 100 to 700 µL of CT solution were combined with 250 µL Buffer A (0.20 M acetic acid, 0.17 M NaCl, pH 4.9), 50 µL bovine serum albumin (BSA) (5 mg/mL in Buffer A) and brought to a final volume of 1 mL with deionized water, and incubated at room temperature for 30 min before centrifuging at 16,000 x g for 5 min at 4°C. Supernatants were removed by vacuum aspiration and the protein-phenolic pellet was washed with 100 µL Buffer A before re-centrifuging and aspirating. Pellets were dissolved in 800 µL of sodium dodecyl sulfate (1% w/v)-triethanolamine (5% v/v) before adding 200 µL of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 M HCl). Absorbances at 510 nm were read after 15 min.

To determine PB, the pellet was vortexed in 500 µL Buffer A and the solution was transferred into a pre-weighed foil cup and allowed to dry. The dried protein-phenolic residue was analyzed for N using an Elementar Vario Macro C:N analyzer (Elementar Americas, Inc., Mt. Laurel, NJ). Percent N was converted to protein by multiplying it by 6.25.

Statistical Analyses

The GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC, USA) was used for statistical analysis of levels of CT in each species. The model included the main effect of plant species while ECT, PBCT, FBCT, and total CT (TCT) were included as dependent variables. Least Square means were estimated using the LSMEANS statement and when significant effects were detected in the model ($P \leq 0.05$) the LINES option was used for mean separation. The GLIMMIX procedure of SAS was used for statistical analysis of PPP and protein precipitated (PB and PB to PPP ratio). Replicates consisted of three separate plant tissue extracts, on which duplicate lab analyses were performed. The model included the main effect of plant species on the dependent variables PPP, PB and PB:PPP. Least Square means were estimated using the LSMEANS statement and when significant effects were detected in the model ($P \leq 0.05$) the LINES option was used for mean separation. PROC REG of SAS was used for regression analysis.

Results and Discussion

The concentrations of TCT in warm-season perennial legumes (Table 2.1) ranged from 2.4% to 12.5%, with the greatest concentrations found in *D. paniculatum, M. strigillosa,* and *L. stuevei*. *Arachis glabrata*, a non-CT negative control, was confirmed to be CT free. The apparent weight-average molecular weights of CT purified from these legumes, relative to polystyrene standards, ranged from 552 to 1483 Da (Table 2.1).

The two most widely used methods for establishing the molecular weight of CT are thiolysis followed by quantitative HPLC analysis of the extender and terminal units

(Li et al. 2010) or GPC (Bae et al. 1994). Thiolysis provides accurate estimates of the mean degree of polymerization but not the molecular weight distribution (Kennedy & Taylor 2003). GPC has been limited by lack of well-characterized standards that have chromatographic behavior similar to CT (Stringano et al. 2011). Preliminary analysis of the CT from the forage legumes used in this study revealed that several of the samples were not efficiently depolymerized under standard thiolytic conditions (unpublished data), suggesting either unusual extender units (Venter et al. 2012) or recalcitrant interflavan bonds (Yokota et al. 2013). Accurate degree of polymerization cannot be established by thiolysis if the reaction is incomplete, so GPC was chosen to determine molecular weights for the CT from the forage legumes surveyed.

Conditions and standards similar to those used by Huang et al. (2010) were used for GPC to establish relative molecular weights for the CT from the plants surveyed, which ranged from about 600 to 1500 Da, with similar ranges for either M_n or M_w (Table 2.1). The estimated degree of polymerization based on these relative molecular weights would be only 2-4 for a typical procyanidin or prodelphinidin. However, the extraction and purification method could have eliminated oligomers via the Sephadex LH20 sorption chromatography step (Salminen et al. 2011), resulting in low estimates of M_W. Potential problems that arise with the use of GPC methods for polyphenols including CT have recently been reviewed (Stringano et al. 2011). First, polyphenols may interact with chromatographic stationary phases such as PL-gel, leading to overestimates in molecular weight (Williams et al. 1983). Second, calibration plots generated using commercially available standards such as polystyrene tend to underestimate CT sizes (Li

et al. 2010). Finally, the relationship between hydrodynamic volume and molecular weight appears to be a complex function of the subunit composition for different polyphenols, so even with CT standards it is difficult to generate a reliable calibration plot (Kennedy & Taylor 2003). Partial solutions to these problems include using complex mobile phases for GPC (Kennedy & Taylor 2003) and polyphenols from diverse sources to generate calibration plots (Stringano et al. 2011) but it was concluded that even with these approaches, GPC provides an estimate of relative molecular weight and is an acceptable method for determining relationships between molecular weight and biological activity.

Protein precipitable phenolics, PB and PB:PPP in legume CT extracts were assessed (Table 2.1). The plant species surveyed could be divided into three groups based on the PB. Six species, *M. strigillosa, N. lutea, L. cuneata, D. illinoensis, A. angustissima STX* and *L. stuevei*, had PB about 17 times the PB of *L. retusa*. The other two tannin-containing species had PB about 15 times that of *L. retusa*. It is possible that these three groups of plants with high, moderate and low PB might have high, moderate and low biological values, respectively, using measures related to ruminal escape protein.

The values for PPP were parallel to the values for PB, with just a few large exceptions. Similar to PB, *L. retusa* had the least PPP; about 15 times less than the PPP for the moderate PPP group. Similar to PB, *L. stuevei* had a very high PPP, but *D. paniculatum*, which had a moderate PB, had the highest PPP. *Mimosa strigillosa* had a high PPP despite having the least PB of the high PB group. The range of values for the

Table 2.1. Percent condensed tannins (ECT: extractable condensed tannins; PBCT: protein-bound condensed tannins; FBCT: fiber-bound condensed tannins; TCT: total condensed tannins), condensed tannin molecular weights (M_n: number-average molecular weight; M_W: weight-average molecular weight), and condensed tannin protein precipitating ability (PPP: protein-precipitable phenolics, g/kg dry matter; PB: amount of protein bound, g/kg dry matter; PB:PPP: ratio of amount of protein bound to protein precipitable phenolics) from warm-season perennial legumes.

Plant	ECT	PBCT	FBCT	TCT	M_n	M_W	PPP	PB	PB:PPP
Desmanthus illinoensis	5.1^{c}	2.4^{bc}	0.6^{a}	8.1^{b}	840	866	54.1^{f}	75.3^{bc}	1.39^{a}
Desmodium paniculatum	10.3^{a}	2.0^{cd}	0.1^{cd}	12.5^{a}	583	1039	200^{a}	64.7^{d}	0.32^{cd}
Lespedeza cuneata	4.7^{cd}	3.4^{a}	0.1^{cd}	8.3^{b}	1276	1483	57.7^{f}	78.1^{ab}	1.36^{a}
Lespedeza stuevei	9.9^{a}	1.7^{cde}	0.1^{cd}	11.7^{a}	520	552	168^{b}	72.3^{c}	0.43^{cd}
Leucaena retusa	2.4^{e}	0.7^{ef}	0.2^{cd}	3.2^{c}	901	950	6.0^{g}	4.4^{e}	0.74^{bc}
Mimosa strigillosa	9.9^{a}	1.7^{cd}	0.2^{cd}	11.7^{a}	768	820	125^{c}	70.9^{c}	0.57^{bc}
Neptunia lutea	7.0^{b}	1.2^{def}	0.1^{cd}	8.3^{b}	1154	1179	82.0^{e}	80.0^{a}	0.98^{ab}
Arachis glabrata	0.0^{g}	0.0^{f}	0.0^{d}	0.0^{d}	819	851	0.0^{f}	0.0^{e}	0.0^{d}
Acacia angustissima var *hirta* (STX)[2]	4.9^{cd}	3.3^{ab}	0.3^{bc}	8.5^{b}	1099	1132	118^{d}	73.7^{bc}	0.63^{bc}
Acacia angustissima var *hirta* (STP5)[1]	4.4^{d}	4.0^{a}	0.5^{ab}	8.9^{b}	1008	1064	87.8^{e}	65.6^{d}	0.75^{bc}

[1]STP5: cross-timbers ecotype
[2]STX: South Texas ecotype
[a-g] Within a column, LS means without a common superscript differ ($P \leq 0.05$).

high PPP species was about 1.7-fold, while the range for PB was only about 1.1-fold, reflecting more variability in PPP than for PB.

The ratio between PB and PPP reveals the efficiency of action by CT, with a large ratio indicating a very efficient protein-precipitating agent. The two species with the largest PB:PPP, *D. illinoensis* and *L. cuneata*, had high PB and moderate PPP, while the species with the smallest ratio, *D. paniculatum*, had moderate PB but very high PPP. The species with the least PPP and PB, *L. retusa*, had an intermediate ratio. At this time, it is not clear whether the PB, the PPP or the ratio more accurately predicts biological activity.

The protein precipitation data was combined with the relative molecular weight data from GPC to test the hypothesis that CT M_W and protein precipitating ability are related. In the present study, there was no correlation between PPP, PB or the ratio PB:PPP and the relative M_W of CT (Fig. 2.1). Condensed tannins extracted from *L. cuneata* had the greatest relative Mw of all species surveyed, had a high PB, but only a moderate PPP. Condensed tannins extracted from *L. stuevei* had the lowest M_W, a high PB and greater PPP than *L. cuneata. Desmodium paniculatum* had the greatest PPP and had a large relative M_W, but had a moderate PB and the smallest PB:PPP. While there was no relationship between relative M_W and PB, PPP, or PB:PPP, *L. cuneata*, had the greatest M_W CT, which was also among the greatest for PB and PB:PPP. The lack of overall correlation suggests that there are other factors involved.

The amount of acid butanol-reactive tannin, TCT, in each plant species had strong-positive correlations with PPP and PB (Fig. 2.2a and 2.2b). *Desmodium*

paniculatum, had the greatest TCT and PPP but moderate PB, whereas *L. retusa* had the least TCT, PPP and PB. The correlations of PB and PPP with TCT agree with Tharayil et al. (2011) who reported positive correlations between CT and protein precipitation for extracts of leaf litter from *Acer rubrum*. In contrast however, Martin & Martin (1982) measured the phenolic, proanthocyanidin and protein-precipitating constituents in extracts from foliage of six *Quercus* species and reported no correlation between CT and protein-precipitating capacity. It is possible that the lack of correlation reported was a function of the proanthocyanidin assay used (Hillis & Swain 1959), which may have only accounted for the ECT and not the PBCT or FBCT fractions, or the protein precipitation method used (Hagerman and Butler 1978), which differed from the radial diffusion assay (Hagerman 1987) used by Tharayil et al. (2011).

A second objective of this study was to identify warm-season perennial legumes that produce biologically active CT and potentially increase efficiency of protein utilization by ruminants, with special attention given to the use of North American native species. Four of the seven North American native warm-season perennial legumes included in this study had greater PPP than the introduced species *L. cuneata,* which has proven biological activity. However, only three of the seven native species, *N. lutea, D. illinoensis* and *A. angustissima STX*, had an amount of PB as great as that of *L. cuneata*. Ruminants consuming a diet that includes legumes containing biologically active CT could experience an increase in the overall efficiency of protein utilization due to decreased ruminal protein degradation resulting in more plant protein entering the lower gastrointestinal tract (GIT) and increased intestinal absorption of amino acids

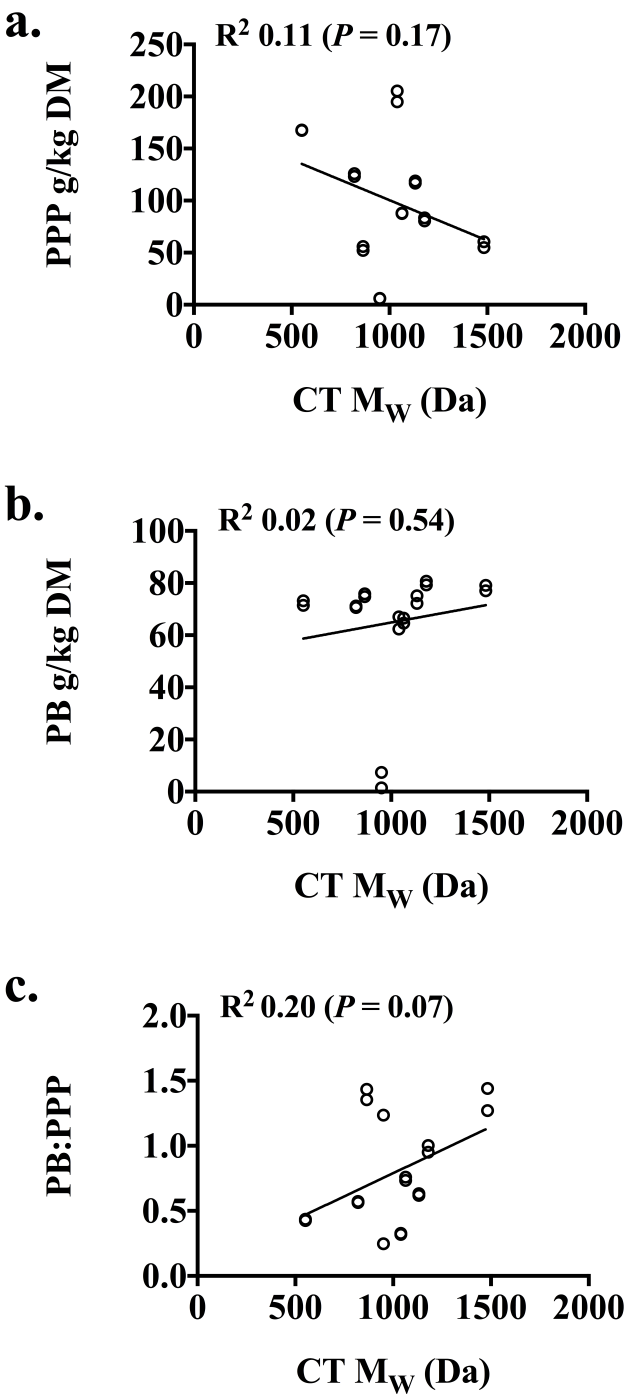

Fig. 2.1. Effects of weight-average molecular weight (M_W) of condensed tannins from warm-season perennial legumes on: a. protein-precipitable phenolics (PPP g/kg DM); b. amount of protein bound by protein-precipitable phenolics (PB g/kg DM); c. amount of protein bound per g of protein-precipitable phenolics (PB:PPP).

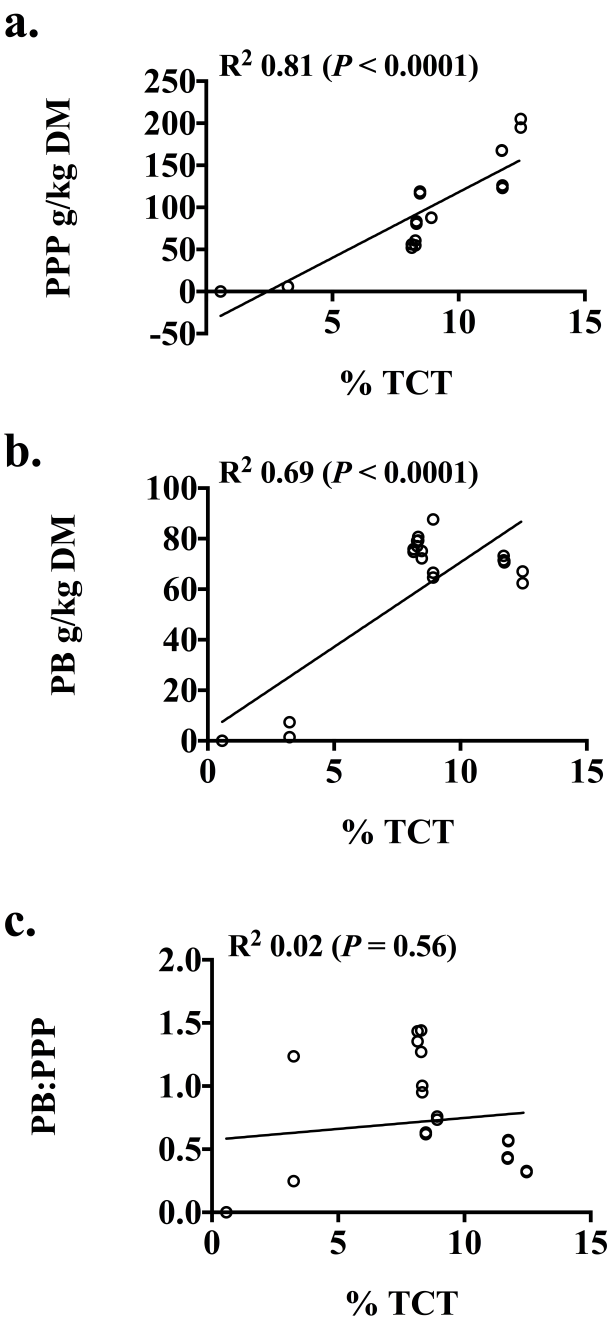

Fig. 2.2. Effects of total condensed tannin concentration (% TCT) on a DM basis of warm-season perennial legumes on: a. protein-precipitable phenolics (PPP g/kg DM); b. amount of protein bound by protein precipitable phenolics (PB g/kg DM); c. amount of protein bound per g of protein-precipitable phenolics (PPP:PB).

(Perez-Maldonado & Norton 1996). An additional advantage is decreased loss of nitrogen (N) as ammonia (NH_3) following ruminal proteolysis and amino acid deamination (Min et al. 2005; Gurbuz et al. 2008). Protein-CT complexes that form in the rumen but dissociate in the abomasum could alter protein digestibility. Spalinger et al. (2010) reported that feeding plants containing CT reduced protein digestibility in moose by 38%. However, Kariuki & Norton (2008) reported that CT-protein complexes become dissociated in the GIT, where protein degradation and subsequent amino acid absorption can occur. It is possible that the reduction in protein digestibility observed by Spalinger et al. (2010) was a result of CT re-complexing with endogenous proteins in the lower GIT due to the lack of any mechanism for CT to undergo digestion in the GIT (Kariuki & Norton 2008).

Condensed tannin-protein complexes may also cause a shift in N excretion from urinary ammonia (NH_3-N), which has potentially detrimental effects on the environment, to fecal N. Perez-Maldonado & Norton (1996) reported that in sheep and goats fed diets containing *D. intortum* or *C. calothyrsus* containing TCT at 0.95 and 2.25%, respectively, there was as much as a 14% increase in fecal N excretion. In agreement, Dschaak et al. (2011) reported that in lactating dairy cows supplemented with *Schinopsis* spp. (Quebracho) tannin extract at a concentration of 3% of the diet there was a shift in N excretion. It is possible that N absorption by ruminants consuming CT is decreased, resulting in apparent shifts from urinary N to fecal N. However, Perez-Maldonado & Norton (1996) reported 19% greater absorption of N in sheep and goats fed diets containing CT.

In regards to increasing efficiency of protein utilization and potentially altering the form of N excreted by ruminants from that of urinary NH_3 to fecal N, which is often incorporated into bacterial proteins, results from the present study indicate that ruminants would have to consume 1.2 times as much *D. paniculatum* or *A. angustissima* STP5 to achieve similar PB to that of *L. cuneata* or *N. lutea*. This may be a function of the protein-binding capacity of CT. The protein-binding capacity of CT from *L. cuneata* was more than four times greater than that of *D. paniculatum*, which had a PPP concentration almost four times greater than *L. cuneata*. Differences in protein binding capacity of CT could translate into differences in biological value of forages consumed by ruminants, especially as it relates to ruminal escape protein.

Conclusions

The tannins in the plant species examined here were all CT, which led to initial speculation that there might be a strong relationship between relative molecular weight and protein precipitation. The data do not support that hypothesis. Preliminary structural analysis using acid butanol or thiolysis to provide monomer composition suggests that although the tannins from these plants are all CT, there is significant structural diversity among them (unpublished data). As a result, it will be necessary to elucidate structural details to understand the molecular basis for different protein precipitating abilities of the CT from these plant species.

North American native warm-season perennial legumes that have promise for use in ruminant diets for the purpose of binding proteins were identified. Use of these legumes in ruminant production could potentially increase efficiency of protein

utilization by ruminants and alter the form of N excreted. Further *in vivo* evaluations of *L. stuevei*, *M. strigillosa N. lutea, D. illinoensis, D. paniculatum*, and two ecotypes of *A. angustissima* var. *hirta* are warranted to determine if *in vitro* and *in vivo* data are correlated and to identify which species truly increase protein utilization by ruminants.

CHAPTER III

EFFECT OF MOLECULAR WEIGHT OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES ON RUMINAL METHANE PRODUCTION *IN VITRO*[*]

Introduction

Interest in the use of native warm-season perennial legumes, as opposed to introduced species, for ruminant production is increasing (Muir et al., 2005). This is especially true in situations where self-sustaining, more stable mixed pastures and rangelands are desired (Muir, 2011). However, condensed tannins (CT) can accumulate to a significant level in some legumes (Wolfe et al., 2008), having anti-nutritional effects (Barry and Mcnabb, 1999; Krueger et al., 2010) on ruminants.

Condensed tannins exhibit multiple biological activities in ruminants, including protein binding (Jones and Mangan, 1977), anthelmintic activity (Athanasiadou et al., 2001) and reduction in enteric methane (CH_4) emission (Tedeschi et al., 2011). Results from previous studies suggested that feeding forages that contain bioactive CT to ruminants effectively inhibits CH_4 produced during enteric fermentation (Huang et al., 2011; Pellikaan et al., 2011; Puchala et al., 2012). A major question raised by these reports is: what causes CT from some plant sources to be bioactive (Cenci et al., 2007;

---

[*] Reprinted with permission from "Effect of molecular weight of condensed tannins from warm-season perennial legumes on ruminal methane production *in vitro*" by Naumann, H.D., L.O. Tedeschi, J.P. Muir, B.D. Lambert, and M.M. Kothmann, 2013. *Biochemical Systematics and Ecology*, 50, 154-162, Copyright 2013 by Elsevier Ltd.

Brunet et al., 2008; Terrill et al., 2009), whereas CT from others demonstrate almost no bioactivity (Pomroy and Adlington, 2006; Whitley et al., 2009) in the ruminant animal? Molecular weight could be an important factor in the bioactivity of CT, such that as molecular weight of CT increases, bioactivity also increases (Bate-Smith, 1973; Peleg et al., 1999). However, others have suggested that an increase in molecular weight of CT does not result in increased bioactivity (Huang et al., 2010; Tharayil et al., 2011). The objectives of this study were to determine if the molecular weight of CT from warm-season perennial legumes affects the bioactivity of CT relative to suppression of CH_4 production by ruminants, and to identify potential North American native forage plants to be used for enteric CH_4 emission mitigation in domesticated ruminants. To achieve these objectives, CH_4 production by fermentation of individual warm-season perennial legumes known to contain CT was evaluated.

Materials and Methods

Experimental Forages

The plant material evaluated consisted of leaves from native Texas herbaceous perennial legumes *Leucaena retusa* Benth. (littleleaf leadtree), *Desmanthus illinoensis* (Michx.) MacMill. Ex B.L. Rob. & Fernald (Illinois bundleflower), *Lespedeza stuevei* Nutt. (tall lespedeza), *Mimosa strigillosa* Torr. & A. Gray (powderpuff), *Neptunia lutea* (Leavenworth) Benth. (yellow puff), two ecotypes (STP5 from the Cross-Timbers of Texas and STX from south Texas) of *Acacia angustissima* var. *hirta* (Nutt.) B.L. Rob (prairie acacia), *Desmodium paniculatum* (L.) DC. var. *paniculatum* (panicledleaf

ticktrefoil), collected in Stephenville, Texas, USA (32° 15' N, 98° 12' W, altitude 395 m). *Arachis glabrata* Benth. (rhizoma peanut), included as a negative control because of its typical low CT content, and *Lespedeza cuneata* (Dum. Cours.) G. Don (sericea lespedeza) are introduced species, the latter listed as a noxious weed in Colorado and Kansas but with known bioactive CT. All plants were collected at Stephenville, TX USA on a Windthorst sandy loam soil (Udic Paleustalf; 10 mg/kg nitrate-N, 13 mg/kg P, 206 mg/kg K, 1416 mg/kg Ca, 247 mg/kg Mg, 15 mg/kg S, 152 mg/kg Na, 10.12 mg/kg Fe, 0.59 mg/kg Zn, 1.10 mg/kg Mn, and 0.22 mg/kg Cu using Mehlich III extraction; Mehlich, 1984). Leaves were dried at 55°C in a forced air oven for 48 h, then ground to pass a 1-mm screen in a sheer mill (Wiley Arthur H. Thomas Co., Philadelphia, PA) and stored for subsequent chemical analysis.

Condensed Tannin Quantification

Condensed tannin fraction and concentration were determined for each plant species as described by Terrill et al. (1992). Extractable CT (ECT) were extracted from 250 mg of plant tissue with 10 mL of a 70:30 solution of aqueous acetone followed by 10 mL of diethyl ether. Protein-bound CT (PBCT) were extracted from the ECT residue with 10 mL of sodium dodecyl sulfate-mercaptoethanol. Fiber-bound CT (FBCT) were determined using the residue remaining from ECT and PBCT. Condensed tannin concentrations in each fraction were determined based on absorbance at 550 nm (Thermo Fisher Scientific, Hudson, NH) following reaction with butanol-HCl. Species-specific standards were created for each plant species analyzed (Wolfe et al., 2008) using

CT extracts purified on Sephadex LH-20 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and lyophilized to recover purified CT.

Condensed Tannin Molecular Weight

Molecular weights of CT from each species were determined by Gel Permeation Chromatography (Waters, Milford, MA) as described by Huang et al. (2010) using a PLgel 3-μm Mixed-E column (Agilent Technologies, Santa Clara, CA) and tetrahydrofuran as the mobile phase. Purified ECT were dissolved in tetrahydrofuran to a final concentration of 0.5 mg/mL and 50 μL injected at a flow rate of 1.0 mL/min at 25°C. Relative molecular weights were calculated based on a calibration curve developed using Polystyrene Low EasiVials (4 mL) (Agilent Technologies, Santa Clara, CA, USA) ranging from 162 to 38,640 Da. Relative weight-average molecular weight (M_W), number-average molecular weight (M_n) and polydispersity index (PDI) were calculated. Approximate degree of polymerization (DP) was calculated based on the report by Williams et al. (1983) that a single proanthocyanidin unit has a M_W of approximately 500 Da.

In vitro *Fermentation*

Two replications were made for each plant species. There were two fermentation chambers that were concurrently run on two separate occasions (Replicate 1; Dec 7, 2011 and Replicate 2; Jan 23, 2012). The bottles (fermentation flasks) inside each fermentation chamber were duplicated for each plant species and not considered true replicates. Each fermentation chamber was considered a block (random variable) in our analysis. The bottles within each fermentation chamber were considered random factors.

Methane and total gas production were determined using an *in vitro* gas production technique as described by Tedeschi et al. (2009). For each replicate, two ruminally-cannulated steers un-adapted to forage containing CT and fed bermudagrass (Cynodon dactylon L. Pers.) hay were used for rumen fluid collection. Rumen fluid was collected concurrently from both steers and mixed at the time of collection. The same animals, consuming the same bermudagrass diet, were used for both replicates. With the exception of non-substrate containing blanks, a subsample (200 mg) of each forage species was transferred to a 150-mL Wheaton bottle, which served as the fermentation flask. A standard diet of Alfalfa (*Medicago sativa* L.) was included as a laboratorial control. Rumen fluid was filtered through cheesecloth to contain large particulate matter followed by glass wool to filter out small particulate matter while continuously mixing with CO_2 to maintain an anaerobic environment. A total of 14 mL of pH stabilized (6.8-6.9) CO_2 ventilated phosphate-bicarbonate media (Goering and Van Soest, 1970) and 2 mL of boiled distilled water were added to the incubation flask containing the plant material. Flasks were sealed with greased butyl rubber stoppers and crimp sealed. A 4-mL subsample of mixed rumen fluid was injected into each sealed flask and incubated in each of two fermentation chambers once they reached a temperature of 39°C. Pressure sensors were attached to each flask and the pressure within each flask was returned to zero by puncturing with a needle. Pressure readings were made automatically each 5 min for a period of 48 h. Pressure data were recorded using Pico Technology software (Eaton Socon, Cambridgeshire, UK). After 48 h of fermentation, incubation flasks were removed from the fermentation chamber and placed in an ice bath to halt any further gas

production. Upon completion of the 48-h fermentation, individual gas samples, a mixture of the gasses produced during the 48-h fermentation, were taken from the headspace of individual fermentation flasks using a 10-mL gas tight syringe. Methane gas measurements were made by gas chromatography (Gow Mac Instrument Co., Bethlehem, PA) using a 5% CH_4 standard.

Volatile fatty acid production was determined by the MixAlco Process™ (Holtzapple et al., 1999), which measures carboxylic acids in liquid process samples. Briefly, liquid subsamples were transferred from the Wheaton flasks to microfuge tubes following 48 h of fermentation and immediately frozen at -20°C for later analysis. Just prior to analysis, samples were thawed, vortexed and centrifuged at 4000 rpm for 5 min at 5°C. A 0.5-mL aliquot of the resulting supernatant from each sample was transferred into individual 2-mL microfuge tubes containing 0.5 mL of 4-methyl-valeric acid as an internal standard and 0.5 mL of 3-*M* phosphoric acid. Microfuge tubes were capped to prevent evaporation of the volatile acids before analysis and centrifuged at 12,000 rpm for 1 min at 5°C. A 1-mL volume of the resulting sample was transferred into a gas chromatograph (GC) vial and capped for analysis.

Volatile fatty acids were measured using an Agilent 6890 GC (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector and 7683 series injector. Conditions for acid analysis were as follows: Inlet conditions included a temperature of 230°C, pressure of 15 psig and gas flow of 185 mL/min. Detector conditions included a temperature of 230°C, an air flow rate of 400 mL/min, a H_2 flow rate of 40 mL/min and a He flow rate of 45 mL/min. Oven conditions included an initial temperature of 40°C,

an initial hold time of 2 min, a ramp rate of 20°C/min, a final temperature of 200°C and a final hold time of 1 min.

Statistical Analyses

Data were analyzed using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC, USA). The model for CT fraction concentration isolated from each species contained the effects of plant species and ECT, PBCT, FBCT, and TCT were included as dependent variables. Least Square means were estimated using the LSMEANS statement and when significant effects were detected in the model ($P \leq 0.05$) the LINES option was used for mean separation. The model for dependent variables CH_4, total gas production and VFA contained the effects of species upon confirming there was no species by fermentation chamber interaction. An incomplete block design was assumed where fermentation chamber was a block and plant samples (species) were fermented in each chamber for as many as four fermentations. Plant samples were fermented in duplicate for each block. Least Square means were estimated using the LSMEANS statement and when significant effects were detected in the model ($P \leq 0.05$) the LINES option was used for mean separation. PROC REG of SAS was used for regression analyses. A probability of $P \leq 0.05$ was considered significant.

Results and Discussion

Condensed Tannin Concentration and Molecular Weight

The fractional concentrations of CT determined by Butanol-HCl method (Terrill et al., 1992) from native warm season perennial legumes are listed in Table 3.1. The

Table 3.1. Concentrations (% DM) of condensed tannin fractions of warm-season perennial legumes.

Plant	ECT[1]	PBCT[2]	FBCT[3]	TCT[4]
Desmanthus illinoensis	5.146^{c}	2.379^{bc}	0.624^{a}	8.149^{b}
Desmodium paniculatum	10.339^{a}	2.047^{cd}	0.078^{cd}	12.463^{a}
Lespedeza cuneata	4.741^{cd}	3.442^{a}	0.112^{cd}	8.295^{b}
Lespedeza stuevei	9.948^{a}	1.687^{cde}	0.071^{cd}	11.707^{a}
Leucaena retusa	2.388^{e}	0.673^{ef}	0.183^{cd}	3.245^{c}
Mimosa strigillosa	9.869^{a}	1.724^{cd}	0.151^{cd}	11.744^{a}
Neptunia lutea	7.023^{b}	1.179^{def}	0.130^{cd}	8.332^{b}
Arachis glabrata	0.332^{g}	0.217^{f}	0.021^{d}	0.569^{d}
Acacia angustissima **var** ***hirta*** **(STX)[5]**	4.898^{cd}	3.278^{ab}	0.290^{bc}	8.466^{b}
Acacia angustissima **var** ***hirta*** **(STP5)[6]**	4.397^{d}	3.977^{a}	0.544^{ab}	8.918^{b}

[1]ECT: extractable condensed tannins
[2]PBCT: protein bound condensed tannins
[3]FBCT: fiber bound condensed tannins
[4]TCT: total condensed tannins.
[5]STX: South Texas ecotype
[6]STP5: Cross-timbers ecotype
[a-f] Within a column, means without a common superscript differ ($P \leq 0.05$).

species analyzed could be categorized into three groups for TCT. *Desmodium paniculatum*, *L. stuevei*, and *M. strigillosa* were high CT, ranging from 11.7 to 12.5%. *Desmanthus illinoensis*, *L. cuneata*, *N. lutea*, and two ecotypes of *A. angustissima* var. *hirta* had moderate CT, ranging from 8.1 to 8.9%. *Leucaena retusa* and *A. glabrata* were low CT, measuring 3.2 and 0.5%, respectively.

Molecular weights, PDI, and DP of CT extracted and purified from warm season perennial legumes, relative to polystyrene standards, are shown in Table 3.2. The M_n represents the statistical average molecular weight of all polymer chains in the CT sample. The M_W is based on the molecular size or weight each polymer contributes to the M_W. The PDI (M_W /M_n) represents the breadth of the molecular weight distribution of the polymer. The DP is the estimated number of monomers that make up the polymer of the purified CT. The greatest M_W of CT observed was from *L. cuneata* and measured 1483 Da, whereas CT from *L. stuevei* had the smallest M_W at 552 Da.

In vitro *Gas Production*

In vitro CH_4 production and *in vitro* total gas production by fermentation warm-season perennial legumes are shown in Fig. 3.1 and 3.2, respectively. The CT produced by some of the legumes surveyed had a high degree of biological activity resulting in methane production less than that of non-substrate containing fermentations (data not shown). As a result, CH_4 values were not blank-corrected to account for CH_4 produced in the absence of substrate. The *in vitro* CH_4 production was greatest for *L. retusa* and *A. glabrata* at 40.7 mg/g DM and 38.2 mg/g DM, respectively. The least amount of *in vitro* CH_4 was produced by the two ecotypes (STP5 and STX) of *A. angustissima* var. *hirta*,

Table 3.2. Relative number average molecular weight (M_n), weight average molecular weight (Mw), polydispersity index (PDI), and approximate degree of polymerization (~DP) of condensed tannins from warm-season perennial legumes.

Plant	Mn	Mw	PDI	~DP[1]
Desmanthus illinoensis	840	866	1.03	2
Desmodium paniculatum	583	1039	1.33	2
Lespedeza cuneata	1276	1483	1.16	3
Lespedeza stuevei	520	552	1.06	1
Leucaena retusa	901	950	1.05	2
Mimosa strigillosa	768	820	1.07	2
Neptunia lutea	1154	1179	1.02	2
Arachis glabrata	819	851	1.04	2
***Acacia angustissima* var *hirta* (STX)[2]**	1099	1132	1.03	2
***Acacia angustissima* var *hirta* (STP5)[3]**	1008	1064	1.06	2

[1] ~DP= based on a single proanthocyanidin unit measuring approximately 500 Da (Williams et al., 1983).
[2]STX: South Texas ecotype
[3]STP5: Cross-timbers ecotype

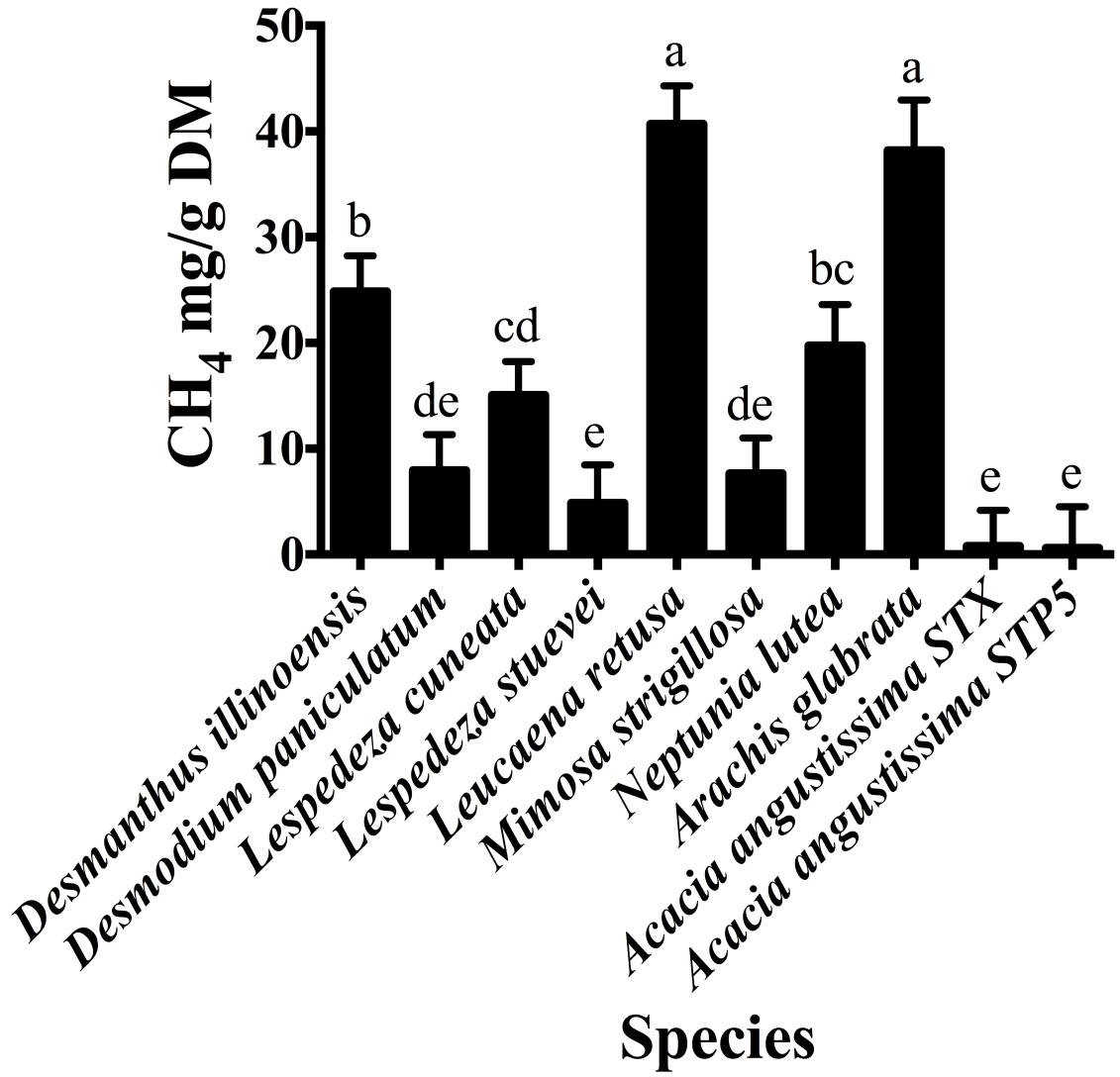

Fig. 3.1. *In vitro* methane production by fermentation of warm-season perennial legumes. Column means without a common superscript differ ($P \leq 0.05$).

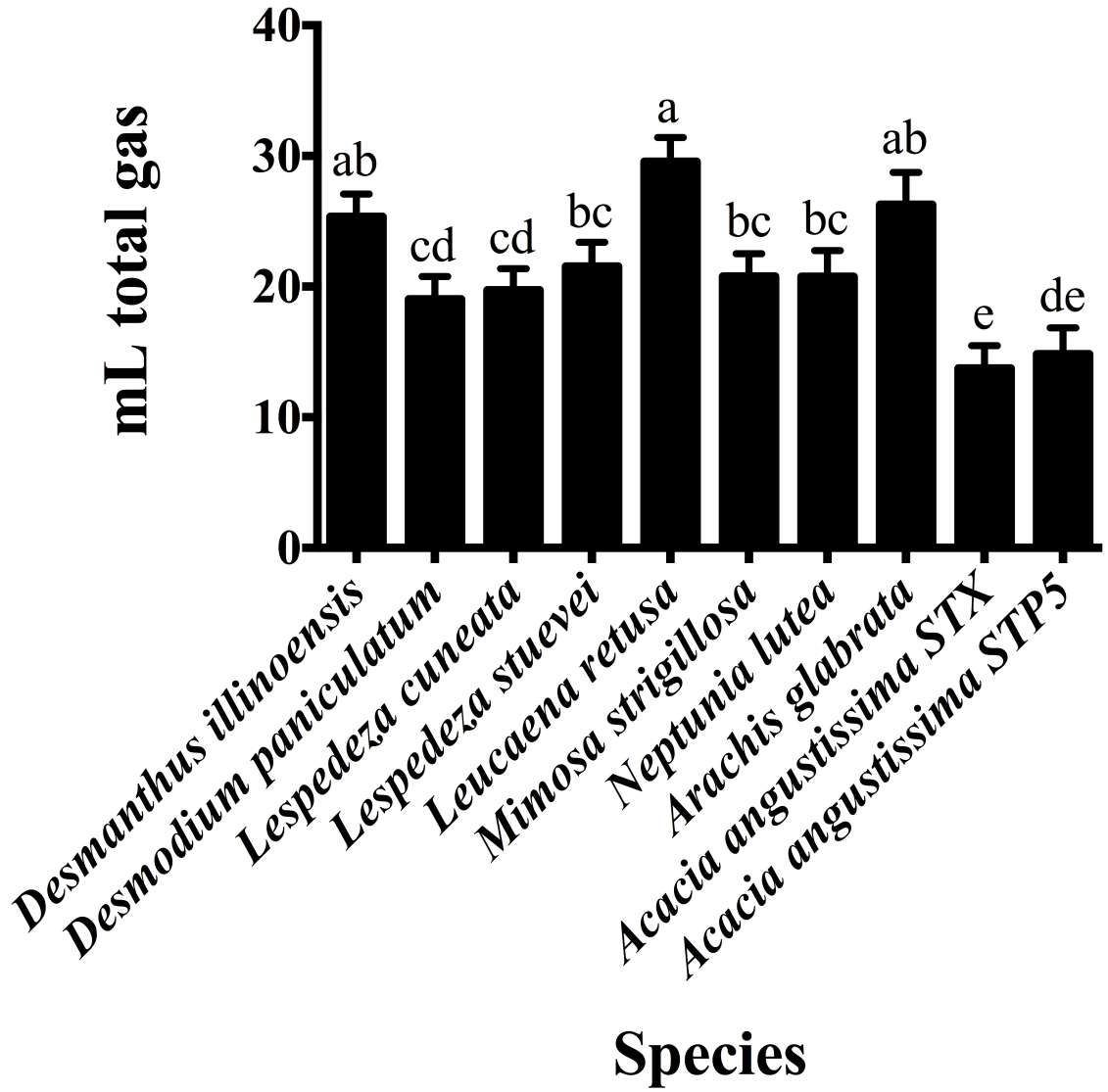

Fig. 3.2. *In vitro* total gas production by fermentation of warm-season perennial legumes. Column means without a common superscript differ ($P \leq 0.05$).

which measured 0.8 and 0.6 mg/g DM, respectively. *In vitro* total gas production by warm season perennial legumes ranged from 29.6 to 13.8 mL. *Leucaena retusa* produced the greatest volume of total gas during *in vitro* fermentation, whereas *A. angustissima* var. *hirta* (STX) produced the least. There was no difference in CH_4 suppression among *D. paniculatum, L. stuevei, M. strigillosa* and the two ecotypes of *A. angustissima* var. *hirta*, which suppressed *in vitro* CH_4 production to the greatest degree. Weight average M_W of these species, which varied by as much as 580 Da, measured 1039, 552, 820, 1132, and 1064 Da, respectively. Fermentation of *L. cuneata*, the species with the greatest ECT M_W, resulted in greater CH_4 production than that of *L. stuevei* and two ecotypes of *A. angustissima*. Fermentation of *A. glabrata*, the low CT negative control, and *L. retusa*, which had ECT M_W of 851 and 950 Da, respectively, resulted in the greatest amounts of CH_4 production despite having greater ECT M_W than *L. stuevei* and *M. strigillosa*.

Of the seven native North American warm-season perennial legumes included in this study, five resulted *in vitro* CH_4 production levels equal to or less than that of the introduced species *L. cuneata*. Only *D. illinoensis* and *L. retusa* resulted in greater CH_4 production than *L. cuneata*. Compared to *A. glabrata*, a forage legume that contains almost no CT, *L. cuneata* produced 60.6% less CH_4. *Lespedeza stuevei* and two ecotypes of *Acacia angustissima* var. *hirta* (STX and STP5) produced 87.3, 98.0 and 99.4% less CH_4 than *A. glabrata*, respectively. Puchala et al. (2012) evaluated ruminal CH_4 emissions by Boer-Spanish crossbred goats consuming fresh forage and hay from *Medicago sativa* or *L. cuneata*. Fresh forage and hay from *Lespedeza cuneata* contained

20 and 15% CT respectively. Compared to *Medicago sativa*, a perennial herbaceous legume similar to that of *A. glabrata* in that it contains almost no CT, *L. cuneata* fresh forage and hay produced 26.2 and 28.6% less CH_4, respectively. This is 34.4 and 32.0% less CH_4 suppression, respectively, than that of the *L. cuneata* used in the present study, which only contained 8.3% TCT compared to 20 and 15% TCT of the *L. cuneata* evaluated by Puchala et al. (2012). These results support the findings of Animut et al. (2008) who reported that the impact of TCT on CH_4 is actually greater per unit of TCT as the amount of dietary TCT decreases, suggesting that factors in addition to TCT are involved in determining biological activity.

Volatile Fatty Acids

Volatile fatty acid production during *in vitro* fermentation of warm-season perennial legumes (Fig. 3.3) is described for acetate, propionate, butyrate, and total VFA. Regressions of CH_4 on C2:C3, CH_4 on total VFA and VFA on TCT are shown in Fig. 3.4. Acetate production was greatest for *A. glabrata* (2.96 g/L) and least for *A. angustissima* var. *hirta* (STP5) (1.21 g/L). Propionate production was greatest for *A. glabrata* (1.12 g/L) whereas the least propionate was generated by *D. paniculatum* (0.53 g/L). The greatest amount of butyrate was produced by *A. glabrata* (0.58 g/L) whereas *A. angustissima* var. *hirta* (STP5) produced the least butyrate at 0.12 g/L. Acetate to propionate ratio was greatest for *A. glabrata* (2.62 g/L) and least for *A. angustissima* var. *hirta* (STX) (1.81 g/L). *Arachis glabrata* produced the greatest amount of total VFA at 5.27 g/L, whereas *A. angustissima* var. *hirta* (STP5) produced the least total VFA at 2.02 g/L.

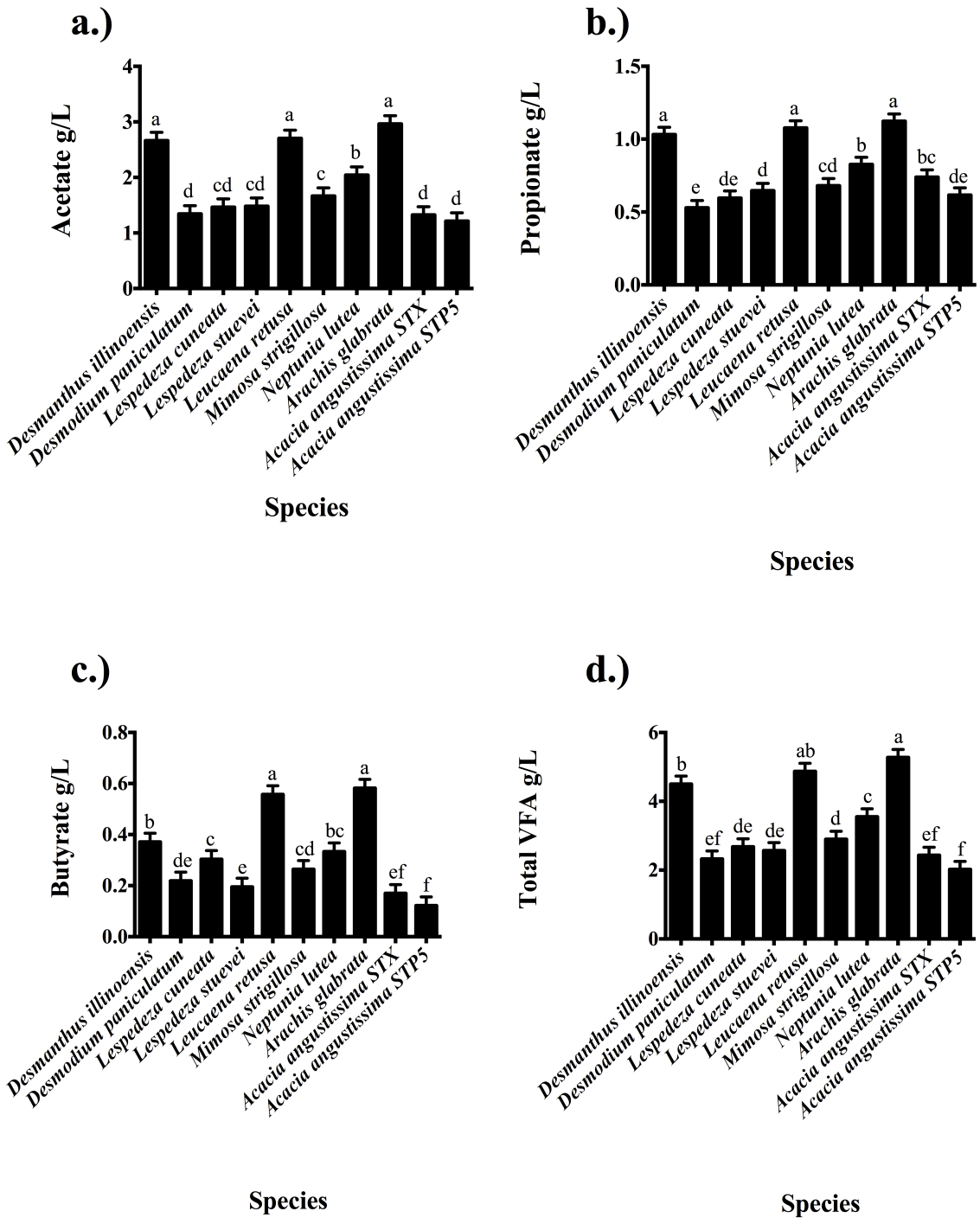

Fig. 3.3. *In vitro* volatile fatty acid production by fermentation of warm-season perennial legumes: a.) acetate production, b.) propionate production, c.) butyrate production, d.) total volatile fatty acid production. Column means without a common superscript differ ($P \leq 0.05$).

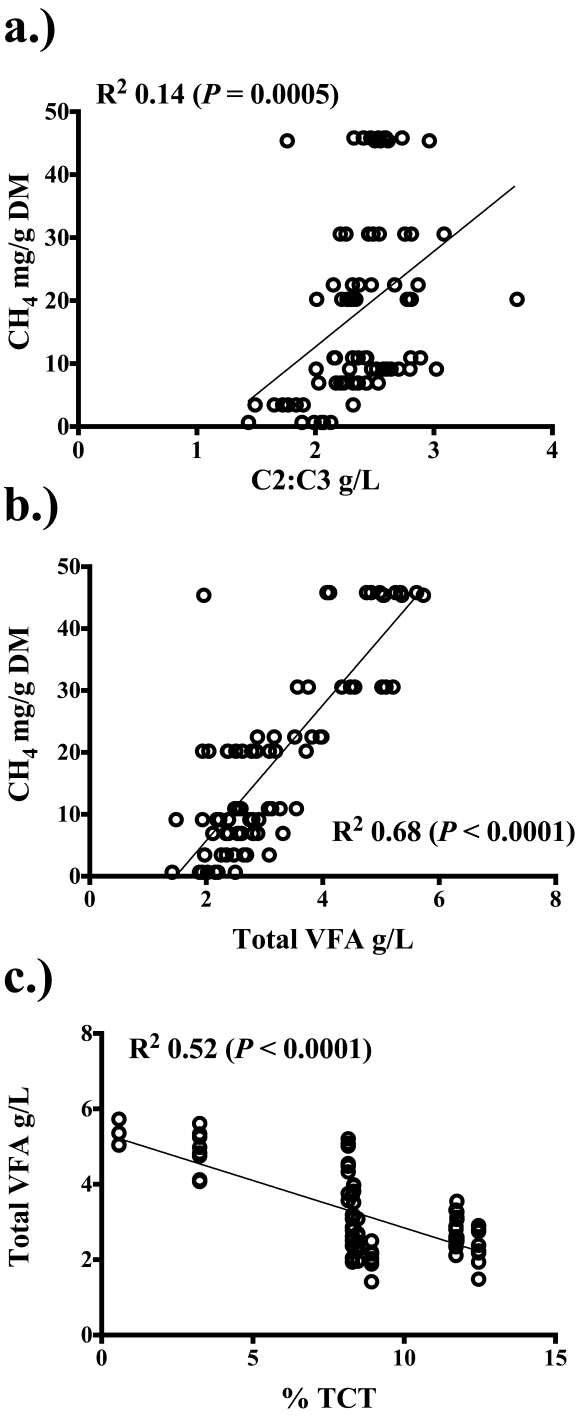

Fig. 3.4. Effects of a.) acetate:propionate and b.) total volatile fatty acid concentration on *in vitro* methane production by fermentation of warm-season perennial legumes; c.) effect of total condensed tannin concentration (percent dry matter) on volatile fatty acid production by *in vitro* fermentation of warm-season perennial legumes.

There are typically strong correlations between enteric CH_4 production and C2:C3 (Russell, 1998; Tavendale et al., 2005). However, there was almost no relationship between *in vitro* CH_4 production and C2:C3 (R^2 0.14; $P = 0.0005$) (Fig. 3.4a). There was a weak-positive correlation between *in vitro* CH_4 and total VFA production (R^2 0.68; $P < 0.0001$) (Fig. 3.4b) in this study. It is possible that the presence of CT in the warm season perennial legumes surveyed interfered with this relationship, as TCT and Total VFA production tended to be related (R^2 0.52; $P < 0.0001$) (Fig. 3.4c). In this model, for every unit increase in TCT, Total VFA production decreased by 0.25 g/L. These results agree with Dschaak et al. (2011), who supplemented dairy cattle diets with CT extract from *Schinopsis* spp. (Quebracho) and reported that total VFA production decreased with CT supplementation.

Molecular Weight of Condensed Tannins and Bioactivity

The effects of ECT M_W and TCT on *in vitro* CH_4 production by warm season perennial legumes are illustrated in Fig. 3.5. A regression of *in vitro* CH_4 production on ECT M_W (Fig. 3.5a.) resulted in an R^2 of 0.0009 ($P = 0.80$), indicating that ECT M_W was unrelated to the amount of *in vitro* CH_4 produced by fermentation of the warm-season perennial legumes surveyed. This result is in agreement with Huang et al. (2010), who suggested that factors other than M_W of CT are involved in determining biological activity based on an evaluation of the protein binding ability of CT of differing M_W from *Leucaena leucocephala* and *Leucaena*-hybrid Bahru. In their case, the smaller M_W CT (2737 Da) from *Leucaena*-hybrid Bahru demonstrated greater biological activity than that of *Leucaena leucocephala,* which measured 2872 Da. This contrasts, however, with

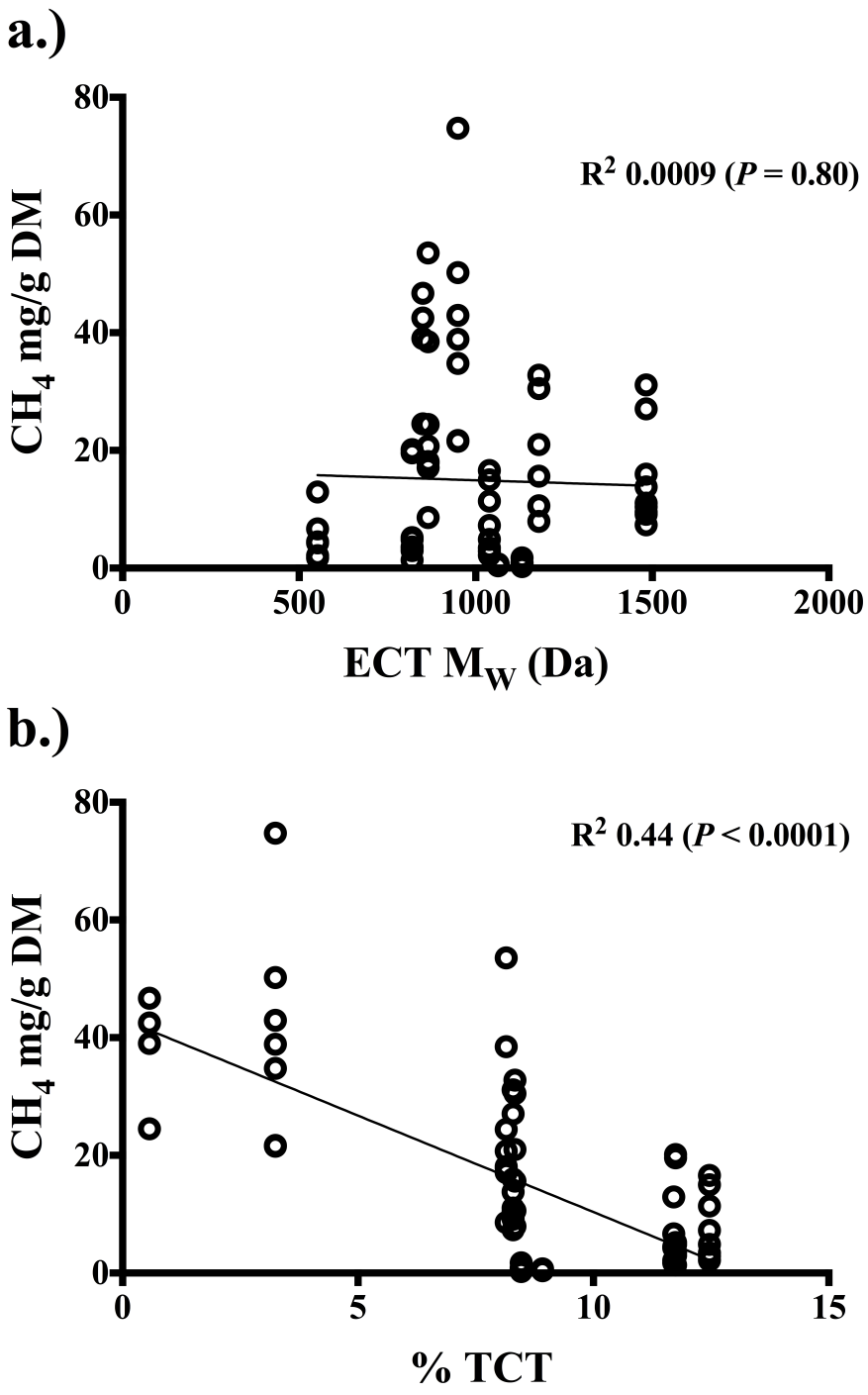

Fig. 3.5. Effects of a.) extractable condensed tannin (ECT) molecular weight and b.) total condensed tannin (TCT) concentration on *in vitro* methane production by fermentation of warm-season perennial legumes.

Huang et al. (2011) who reported a decrease in CH_4 production as M_W of CT increased by using purified ECT M_W fractions isolated from *Leucaena*-hybrid Bahru. It is possible that when evaluating CT alone, the M_W is an influential factor controlling biological activity, but when evaluating whole forage, as would normally be encountered by the ruminant, factors other than M_W have a greater influence on CT biological activity.

A regression of *in vitro* CH_4 production on percent TCT (Fig. 3.5b) resulted in a R^2 of 0.44 ($P < 0.0001$), suggesting that 56% of *in vitro* CH_4 production can be explained by factors other than TCT. For most species surveyed, the greater the concentration of TCT, the greater the CH_4 suppression. However, the two ecotypes of *A. angustissima var. hirta* did not fit this trend. *Acacia angustissima* var. *hirta* (STX) and *A. angustissima* var. *hirta* (STP5) contained 8.47 and 8.92% TCT respectively, which did not differ from that of *D. illinoensis*, *L. cuneata*, and *N. lutea*, which contained 8.15, 8.29 and 8.33% TCT, respectively. However, the two ecotypes of *A. angustissima* var. *hirta* suppressed *in vitro* CH_4 production to a greater degree than *D. illinoensis, L. cuneata*, and *N. lutea*, suggesting that the CT present in *A. angustissima* var. *hirta* could differ characteristically from that of the other species surveyed. This would be in agreement with Pellikaan et al. (2011) who determined that the amount of *in vitro* CH_4 produced depends on the type of CT present in the forage.

The warm-season perennial legumes surveyed varied in ECT M_W, TCT and CH_4 produced during *in vitro* fermentation. The results of this study strongly suggest that ECT M_W does not explain the biological activity of *in vitro* CH_4 production by fermentation of the forage legumes surveyed. However, TCT did explain some of the

variation associated with *in vitro* CH_4 production. It should be noted that M_W was only measured on the ECT and not the PBCT or FBCT. The possibility that the M_W and biological activity of PBCT and FBCT could differ from that of ECT should be explored. To accomplish this, refinement of methodology that will allow for identification of the M_W of PBCT and FBCT is needed. Five of the seven North American native-warm season perennial legumes were identified as having promise for use in ruminant diets for the purpose of CH_4 emission mitigation. Further research exploring the effects of these rangeland legumes on CH_4 production when used together with other feeds is needed.

CHAPTER IV

EFFECT OF MOLECULAR WEIGHT OF CONDENSED TANNINS FROM WARM-SEASON PERENNIAL LEGUMES ON *IN VITRO* LARVAL MIGRATION INHIBITION OF *HAEMONCHUS CONTORTUS*

Introduction

Gastrointestinal nematode (GIN) resistance to a broad spectrum of commercially prepared anthelmintic drugs has been reported as widespread (Prichard, 1990). Until recently, synthetic anthelmintic drugs have been used as the primary control of GIN in U.S. sheep and goat industries (Terrill et al., 2009). While medicinal plants have been used to control GIN as a part of traditional ethnoveterinary practices in rural areas of South Africa for many years (McGaw et al., 2007), the use of plants containing biologically active secondary compounds, specifically condensed tannins (CT), to combat GIN infections in other parts of the world is increasing (Iqbal et al., 2007; Montellano et al., 2010).

Condensed tannins are polyphenolic compounds that may demonstrate biological activities in ruminants including suppression of enteric methane production (Tedeschi et al., 2011), binding to proteins (Jones and Mangan, 1977), and suppression of GIN infections (Athanasiadou et al., 2001). The anthelmintic activity of forage CT is of great interest due to the economic costs of anthelmintic resistant GIN infections to the U.S. sheep and goat industries (Terrill et al., 2009) and elsewhere in the world. Some plant CT are anthelmintically active (Brunet et al., 2008; Marie-Magdeleine et al., 2010),

whereas others have no anthelmintic activity (Pomroy and Adlington, 2006; Whitley et al., 2009). While the chemical structure of CT has been postulated to be a key contributing factor affecting biological activity (Kraus et al., 2003), specifically that of CT-protein interactions (Hagerman and Butler, 1981), the specific factors that determine whether or not forage CT have anthelmintic properties remain unknown. Results from previous studies have shown that as molecular weight of CT increases, CT biological activity relative to CT-protein interactions also increases (Bate-Smith, 1973a; Peleg et al., 1999). Others have reported no correlation of CT molecular weight to biological activity (Huang et al., 2010; Tharayil et al., 2011).

The relationship of molecular weight of CT from a variety of warm-season perennial legumes commonly consumed by sheep and goats to anthelmintic activity has not been previously explored. The objectives of this study were to 1) determine if molecular weight of CT from warm-season perennial legumes could predict the biological activity of CT relative to anthelmintic activity against ivermectin-resistant L3 stage *Haemonchus contortus* (HC), and 2) compare anthelmintic activity of CT from native warm-season perennial legumes to that of the introduced species *Lespedeza cuneata,* a plant that has gained attention in recent years due to its anthelmintic properties (Shaik et al. 2006; Terrill et al. 2009).

Materials and Methods

Experimental Forages

Leaves from native Texas *Leucaena retusa* Benth. (littleleaf leadtree), *Desmanthus illinoensis* (Michx.) MacMill. Ex B.L. Rob. & Fernald (Illinois bundleflower), *Lespedeza stuevei* Nutt. (tall lespedeza), and two ecotypes (STP5: Cross-Timbers of Texas; STX: south Texas) of *Acacia angustissima* var. *hirta* (Nutt.) B.L. Rob (prairie acacia) were collected in Stephenville, Texas, USA (32° 15' N, 98° 12' W, altitude 395 m). *Arachis glabrata* Benth. (rhizoma peanut), included as a negative control because of its typical low CT concentration, and *Lespedeza cuneata* (Dum. Cours.) G. Don (sericea lespedeza) are introduced species that were also collected in Stephenville, Texas. All plants collected at Stephenville were grown on a Windthorst sandy loam soil (Udic Paleustalf; 10 mg/kg nitrate-N, 13 mg/kg P, 206 mg/kg K, 1416 mg/kg Ca, 247 mg/kg Mg, 15 mg/kg S, 152 mg/kg Na, 10.12 mg/kg Fe, 0.59 mg/kg Zn, 1.10 mg/kg Mn, and 0.22 mg/kg Cu using Mehlich III extraction; Mehlich, 1984). Leaves were dried at 55°C in a forced-air oven for 48 h, then ground to pass a 1-mm screen in a sheer mill (Wiley Arthur H. Thomas Co., Philadelphia, PA) and stored for subsequent chemical analyses and LMI assay.

Condensed Tannin Quantification

Condensed tannin fraction and concentration were determined for each plant species as described by Terrill et al. (1992). Extractable CT (ECT) were determined from 250 mg of plant tissue with 10 mL of a 70:30 solution of aqueous acetone followed by 10 mL of diethyl ether. Protein-bound CT (PBCT) were extracted from the ECT

residue with 10 mL of sodium dodecyl sulfate-mercaptoethanol. Fiber-bound CT (FBCT) were determined using the residue remaining from ECT and PBCT. Condensed tannin concentrations in each fraction were determined based on absorbance at 550 nm (Thermo Fisher Scientific, Hudson, NH) following reaction with butanol-HCl. Species-specific standards were created for each plant species analyzed (Wolfe et al., 2008) using CT extracts purified on Sephadex LH-20 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) and lyophilized to recover purified CT.

Condensed Tannin Molecular Weight

Molecular weights of CT from each species were determined by Gel Permeation Chromatography (Waters, Milford, MA) as described by Huang et al. (2010) using a PLgel 3-μm Mixed-E column (Agilent Technologies, Santa Clara, CA) with tetrahydrofuran as a single mobile phase. Purified ECT were dissolved in tetrahydrofuran to a final concentration of 0.5 mg/mL and 50-μL injected at a flow rate of 1.0 mL/min at 25°C. Relative weight-average molecular weights (M_W), defined as the molecular size or weight each polymer contributes to the overall molecular weight, were calculated based on a calibration curve developed using Polystyrene Low EasiVials (4 mL) (Agilent Technologies, Santa Clara, CA, USA) ranging from 162 to 38,640 Da.

In vitro *Larval Migration Inhibition*

Anthelmintic activity of forage CT was determined using an *in vitro* larval migration inhibition (LMI) assay described by Armstrong et al. “In Press”.

Forage

The assumptions made to determine the amount of forage used for plant bioactive extracts included the following: a 45 kg goat consuming 3% of its body weight equaling a total intake of 1,360 g. Of the 1,360 g consumed, an estimated 25% of the diet consumed consisted of a leguminous forage containing CT. A conversion factor of 0.005625 was applied to an assumed average 8 L capacity goat rumen (Whitney et al., 2011) to a 45 mL *in vitro* system. The amount of forage used in each replication was determined on a DM basis using the following formula:

$$Forage = \frac{Goat\ weight\ x\ \%\ forage\ in\ diet\ x\ conversion\ factor}{Forage\ \%\ DM}$$

Larvae

Ivermectin resistant strains of HC L3 stage larvae were coprocultured from fecal material collected from adult Boer-Spanish crossbred wether goats. Fecal material was placed in a humid environment to facilitate development of larvae from L1 stage to infective L3 stage. A 2-mL aliquot of Fungizone (JR Scientific, Woodland, CA USA) was added to the fecal material (5 µg/mL) to prevent fungal growth. Seven to 10 d post incubation, a Baerman apparatus (Dinaburg, 1942) was used to collect infectious L3 larvae. *Haemonchus contortus* was the only species identified and originated from populations resistant to ivermectin (Whitney et al., 2010). Larvae collected were stored in a horizontally vented cap container at a concentration of 2,000 to 2,500 larvae/mL at 10°C and used within 30 d to ensure maximum viability (Todd et al., 1976). Larvae were screened to ensure at least 90% motility prior to use.

Fermentation and condensed tannin extraction

Rumen fluid was collected from mature, ruminally cannulated Boer-Spanish crossbred wether goats and placed in a pre-warmed insulated container. Rumen fluid was strained through six layers of cheesecloth. McDougall's buffer (McDougall, 1947) and rumen fluid were mixed at a ratio of 1:4 to a total liquid volume of 45 mL. Each forage treatment was added individually to a fermentation flask containing rumen fluid and buffer. Flasks were purged with CO_2 and capped with a size 6 rubber stopper (Midwest Brewing Supply Company, Minneapolis, MN USA) and a 3-piece air lock (Midwest Brewing Supply Company, Minneapolis, MN USA), and incubated at 39°C for 18 h to extract CT. Flasks were removed from the incubator and final pH was recorded. A total of 25 mL of each solution were removed and individually centrifuged at 1,912 x g for 15 min. Supernatants containing extracted CT were used to fill well plates.

Larval migration inhibition

Millipore 96 well plates (20 μm screen; Billerica, MA USA) were used to measure LMI of HC for each treatment. The bottom portions of the well plates were filled with 125 μL of treatment supernatant. The screen was placed on the top of the treatment supernatant and gently tapped to remove air bubbles. With screens in place, 10 μL of larvae were placed in each well. An additional 125 μL of treatment supernatant were dispensed into the well to prevent desiccation of larvae. Well plates were sealed and placed in an airtight container purged with CO_2 for 30 s and placed in a shaker incubator at 45 RPM and 39°C for 18 h.

Screens from well plates were removed following incubation. Liquid remaining in the bottom of the well plate was removed using a micropipette and placed on a slide. Larvae were counted under 100x zoom and recorded as migrant larvae. Wells were flushed with 100 µL of water to ensure all larvae that migrated to the bottom of the well plate were counted.

Well plate rows never held more than one treatment type, and equipment was only used for one type of treatment throughout the experiment. A positive control (Ivermectin at 40 µg/mL) and negative control (rumen fluid and buffer) were evaluated along with forage treatments for each replication. *Arachis Glabrata,* a forage legume containing no CT, was used as a negative control for calculating percent LMI. The percent LMI was calculated using the following formula: $percent\ LMI = \left(\frac{A-B}{A}\right) x\ 100$ where A equals the average number of larvae that migrated through negative control wells and B equals the average number of larvae that migrated through the treatment wells.

Statistical Analyses

Three replications of the assay, nine wells per replication, separated by 7 d each and using separate rumen fluid collections were conducted. Data were analyzed using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC, USA). For comparisons of the CT fractions from plants, the model included the main effect of plant species while ECT, PBCT, FBCT, and total CT (TCT) were included as dependent variables. Least Square means were estimated using the LSMEANS statement and, when significant effects were detected in the model ($P \leq 0.05$), the LINES option was used for mean

separation. For percent LMI, the model included the main effect of treatment on the dependent variable percent LMI. Least square means were estimated using the LSMEANS statement and, when significant effects were detected in the model ($P \leq 0.05$), the LINES option was used for mean separation. PROC REG of SAS was used for regression analysis. A probability of $P \leq 0.05$ was considered significant.

Results

Fractional Concentration of Condensed Tannins

The fractional concentrations of CT determined by a modified Butanol-HCl method (Terrill et al., 1992; Wolfe et al., 2008) from warm-season perennial legumes are shown in Table 4.1. *Arachis glabrata*, the CT negative control, was confirmed to be free of CT. The ECT fractions, which accounted for the greatest percentage of the TCT, ranged from 0.0% for *A. glabrata* to 9.9% for *L. stuevei*. The PBCT fractions ranged from 0.0% for *A. glabrata* to 3.9% for *A. angustissima* var. *hirta* (STP5). The FBCT fractions, which accounted for the smallest percentage of the TCT, ranged from 0.0% for *A. glabrata* to 0.6% for *D. illinoensis*. *Lespedeza stuevei* had the greatest TCT, which measured 11.7%. With the exception of *A. glabrata, L. retusa* had the least TCT, which measured 3.3%.

Relative Molecular Weight of Condensed Tannins

Relative M_W of CT extracted and purified from warm-season perennial legumes are shown in Table 4.1. Weight-average molecular weights of CT ranged from 552 to 1,483 Da and were represented by *L. stuevei* and *L. cuneata,* respectively.

In vitro *Larval Migration*

Percent *in vitro* LMI by CT from warm-season perennial legumes is shown in Fig. 4.1. The treatment demonstrating the greatest percent LMI was *L. retusa* (65.4%), which did not differ from that of *L. stuevei* and *A. angustissima* var. *hirta* STP5. The ivermectin treatment had the smallest percent LMI (12.5%) against L3 HC resistant to ivermectin, but did not differ from that of the negative control containing only rumen fluid and buffer, *L. cuneata* and *D. illinoensis*.

CT M_W and Concentration on LMI

The effect of CT M_W on percent LMI is shown in Figure 4.2. There was a weak correlation (R^2 0.34; $P = 0.05$) such that as CT M_W increased, percent LMI decreased. Condensed tannins extracted from *L. cuneata* had the greatest M_W of the species surveyed. However, percent LMI for *L. cuneata* did not differ from that of the control. Condensed tannins from *L. stuevei* and *L. retusa* had M_W of 552 and 950, respectively, both smaller than that of *L. cuneata*. However, percent LMI for *L. stuevei* and *L. cuneata* were greater than for *L. cuneata*. The effects of CT fractional concentrations are shown in Figure 4.3. Some of the variation in percent LMI was explained by the PBCT fraction (R^2 0.48; $P = 0.01$). The ECT, FBCT and TCT resulted in R^2 of 0.03, 0.13 and 0.03, respectively.

Discussion

Identification of the key factors that determine biological activity of CT, specifically that of suppression of GIN parasites, could improve the use of plant species that develop

biologically active CT and foster the development of more efficient commercial products. Results from previous studies (Bate-Smith, 1973; Peleg et al., 1999; Vidal et al., 2003) indicated that M_W of CT may be a contributing factor to CT biological activity. Bate-Smith (1973) used a haemanalysis assay to determine the relative astringency of procyanidins isolated from *Persea gratissima* (avocado) and reported that protein-precipitating ability increased as M_W of the procyanidins increased. Peleg et al. (1999) evaluated the astringency of flavan-3-ol monomers, dimers and trimers and reported an increase in astringency with increased M_W. In a similar study involving proanthocyanidins isolated from *Vitis* and *Malus* species, Vidal et al. (2003) also reported increases in overall astringency as M_W increased.

In contrast, Huang et al. (2010) and Tharayil et al. (2011) reported results which suggested that increased M_W of CT does not contribute to increased biological activity. Huang et al. (2010) evaluated CT from *Leucaena leucocephala* and *Leucaena*-hybrid Bahru, which had M_W of 2872 and 2737 Da, respectively. The smaller M_W *Leucaena*-hybrid Bahru demonstrated greater protein binding ability, suggesting that factors other than M_W determine biological activity of CT. Tharayil et al. (2011) evaluated the effects of climatic stress on reactivity of CT from *Acer rubrum* and reported that CT with lesser degrees of polymerization exhibited the greatest protein-binding ability. In agreement with the latter two studies discussed, results from the present study indicated that factors other than M_W of CT determine, or at least contribute to, the biological activity, specifically that of inhibition of larval migration of L3 stage HC.

Table 4.1. Concentrations (% DM) of condensed tannin fractions and weight-average molecular weights (M_W) of warm-season perennial legumes.

Plant	ECT[1]	PBCT[2]	FBCT[3]	TCT[4]	M_W
Desmanthus illinoensis	5.1^{b}	2.4^{bc}	0.6^{a}	8.1^{b}	866
Lespedeza cuneata	4.7^{bc}	3.4^{a}	0.1^{cd}	8.2^{b}	1483
Lespedeza stuevei	9.9^{a}	1.7^{cde}	0.1^{cd}	11.7^{a}	552
Leucaena retusa	2.4^{d}	0.7^{ef}	0.2^{cd}	3.3^{c}	950
Arachis glabrata	0.0^{e}	0.0^{f}	0.0^{d}	0.0^{d}	0
***Acacia angustissima* var *hirta* (STX)[5]**	5.0^{bc}	3.3^{ab}	0.3^{bc}	8.6^{b}	1132
***Acacia angustissima* var *hirta* (STP5)[6]**	4.4^{c}	3.9^{a}	0.5^{ab}	8.8^{b}	1064

[1]ECT: extractable condensed tannins
[2]PBCT: protein bound condensed tannins
[3]FBCT: fiber bound condensed tannins
[4]TCT: total condensed tannins.
[5]STX: South Texas ecotype
[6]STP5: Cross-timbers ecotype
[a-f] Within a column, means without a common superscript differ ($P \leq 0.05$).

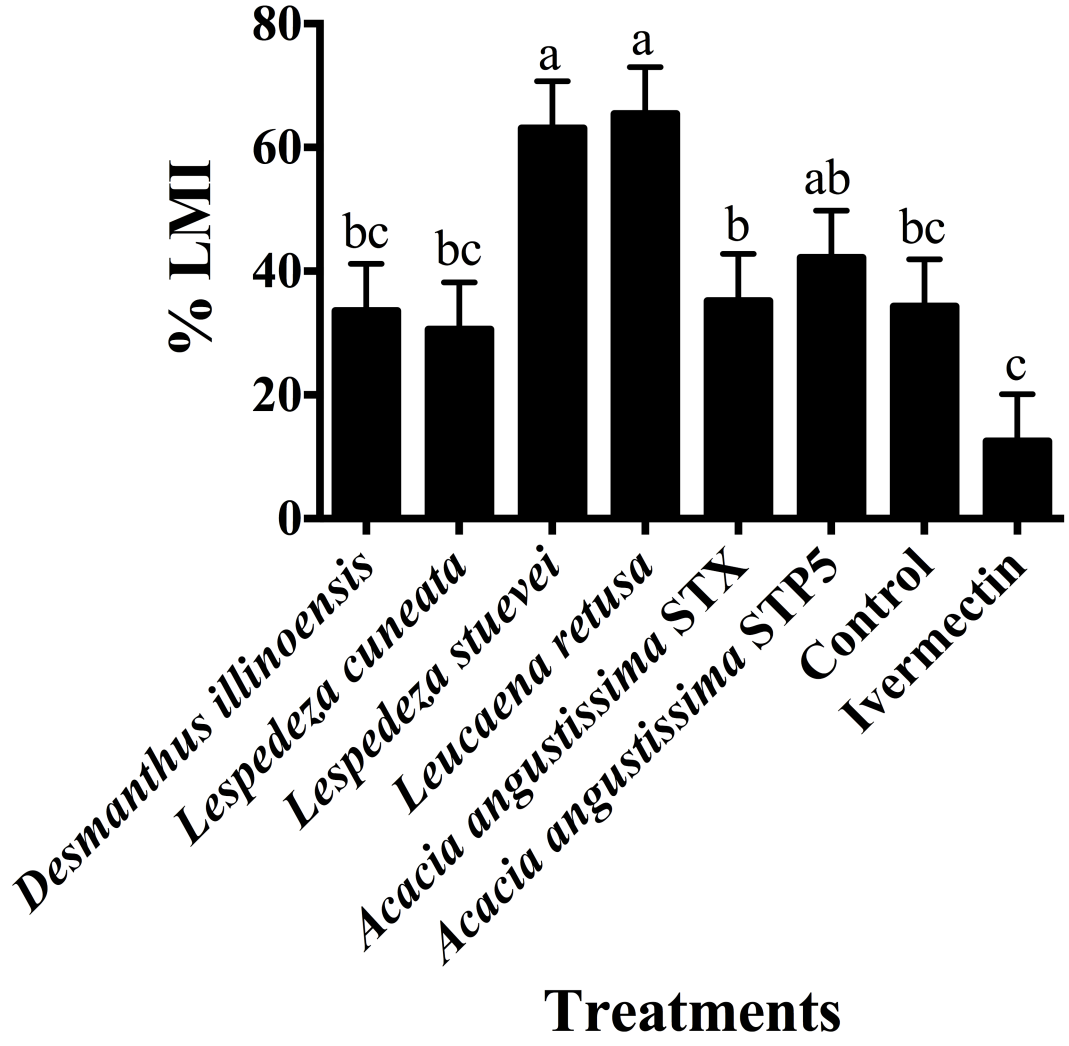

Fig. 4.1. Percent larval migration inhibition of L3 stage *Haemonchus contortus* by condensed tannins from warm-season perennial legumes. Column means without a common superscript differ ($P \leq 0.05$).

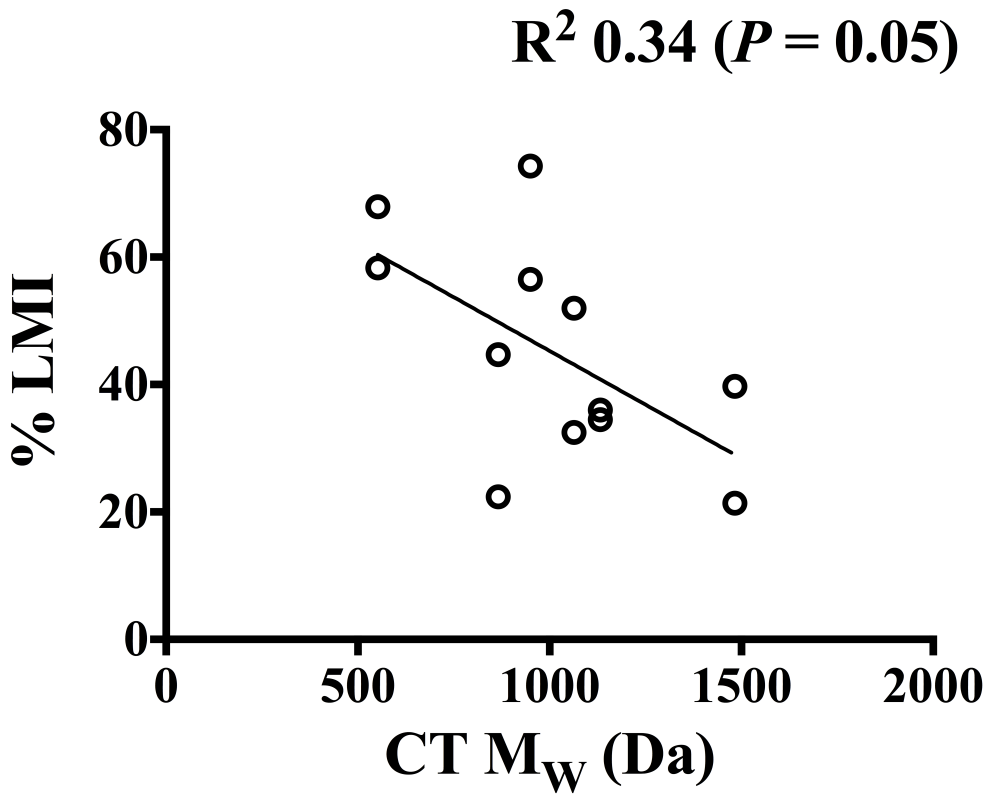

Fig. 4.2. The effect of condensed tannin CT molecular weight (M_W) on percent larval migration inhibition of L3 stage *Haemonchus contortus.*

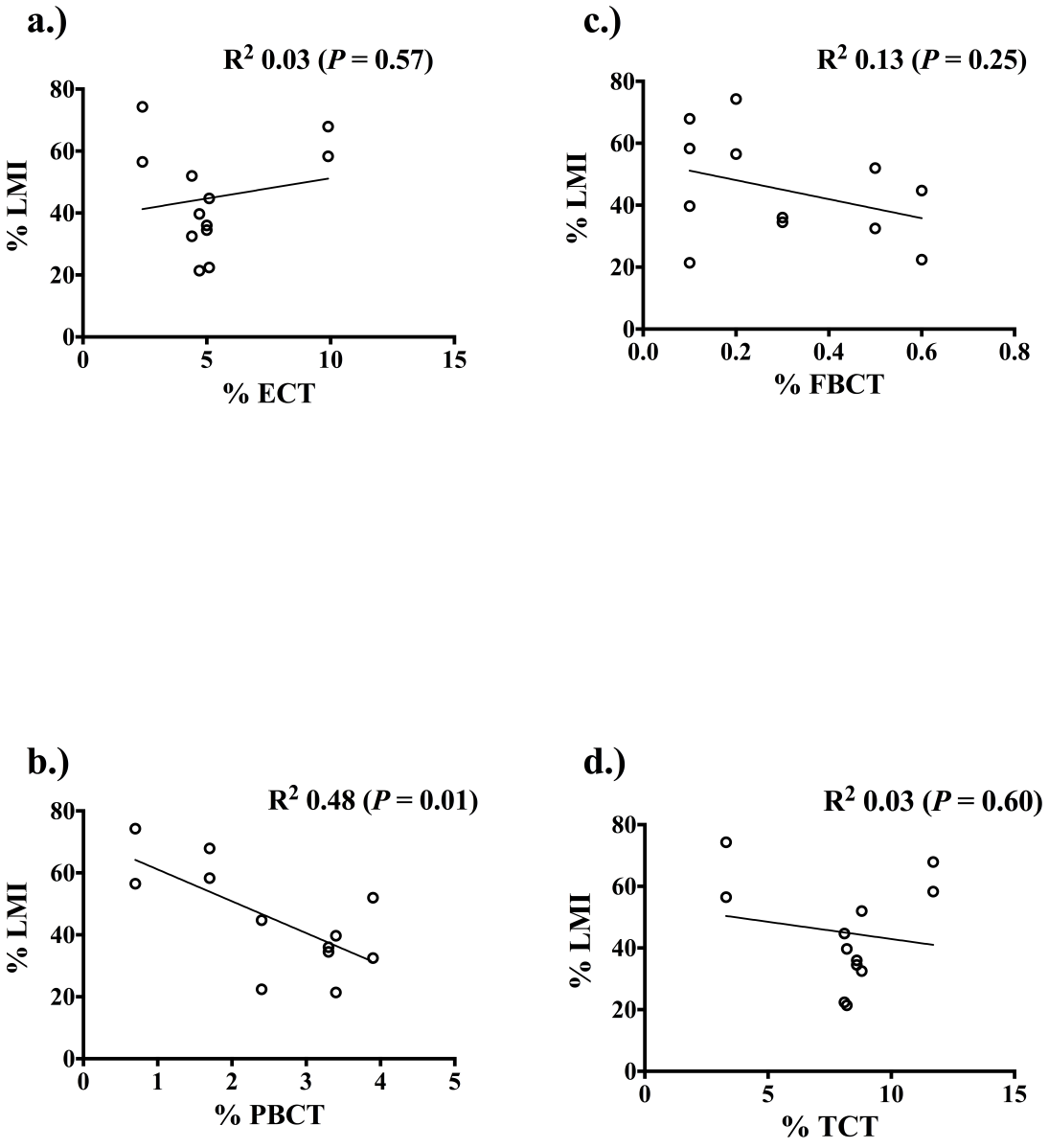

Fig. 4.3. Effects of a.) extractable condensed tannin (ECT) concentration b.) protein-bound condensed tannin (PBCT) concentration c.) fiber-bound condensed tannin (FBCT) concentration and d.) total condensed tannin concentration (TCT; as a percent of dry matter) on percent larval migration inhibition (LMI) of L3 stage *Haemonchus contortus*.

Results from a previous study indicated that the concentration of biologically active CT is a contributing factor in the suppression of adult HC and resulting reductions in fecal egg counts (Terrill et al., 2009). Results from the present study indicated that no relationship exists between the concentrations of biologically active CT and efficacy against L3 stage HC. One possible reason for this disparity is that biologically active CT may not be available to interact with L3 stage HC in the rumen, where L3 stage HC predominate. This is because the activity of CT-protein binding is a pH dependent process (Faithfull, 1984), which results in stable CT-protein complexes at ruminal pH of 6.5 that later become dissociated in the acidic pH found in the abomasum (Jones and Mangan, 1977) where infective adult HC embed into the lining. As a result, biologically active CT present in the rumen may be overwhelmingly bound to proteins and other compounds and unable to affect L3 stage HC. However, after CT complexes become dissociated, which in the case of the ruminant animal occurs in the abomasum where adult HC predominate, they may become available to react once again.

Factors other than CT M_W affecting CT biological activity could involve the more detailed phytochemical aspects of CT structure (Naumann et al., 2013b). For example, hydroxylation of the B-ring of the proanthocyanidin structure could be a contributing factor. A proanthocyanidin consisting of a 3', 4', 5'-tri-hydroxyphenyl is a prodelphinidin unit, whereas a procyanidin consists of a 3', 4'-di-hydroxyphenyl unit (Foo and Porter, 1980; Tharayil et al., 2011). It is possible that the prodelphinidin is more biologically active owing to the additional hydroxyl group. Another factor affecting CT biological activity could be the presence or absence of galloyl groups (Li et

al., 2010), which are flavan-3 ol subunits consisting of tri-hydroxphenyl gallic acid functional groups occurring at the C-3 position of the proanthocyanidin structure (Okuda and Ito, 2011). Stereochemistry of the proanthocyanidin may be either cis or trans depending on the orientation of the functional group located at the C-3 and C-4 positions, and may affect conformational freedom of the proanthocyanidin and subsequent ability to react.

Conclusions

If molecular weight of CT contributes to CT biological activity, its effect on LMI of L3 stage HC is likely connected to other factors and, considered alone, is a weak contributing factor. Further evaluation of the phytochemical characteristics of CT from the warm-season perennial legumes surveyed, relative to percent LMI, is needed to identify possible contributing or synergistic factors. Further study of *L. stuevei* and *L. retusa in vivo* as L3 stage HC suppressors, regardless of mode of action, is warranted.

CHAPTER V

SUMMARY AND CONCLUSIONS

The biological activity of CT relative to suppression of CH_4 emissions, protein binding ability and inhibition of larval migration by L3 stage *Haemonchus contortus in vitro* differ depending on source of CT and are affected to some degree by CT concentration. Condensed tannin M_W does not explain the biological activities of CT relative to *in vitro* CH_4 suppression or protein binding ability. Though weakly correlated, CT M_W may contribute to biological activity relative to inhibition of L3 stage *Haemonchus contortus* migration.

Molecular weight of CT is inclusive of smaller scale molecular characteristics of CT including degree of polymerization, hydroxylation pattern and presence or absence of functional groups. In addition to those associated with CT M_W, characteristics that may affect CT biological activity include stereochemistry and interflavan linkages. The biological activity of CT from forage legumes is likely related to a combination of these characteristics working synergistically to demonstrate biological activity. While concentration explains some but not all of the variation associated with some CT biological activities, in others it explains very little. Furthermore, while concentration is important when dealing with CT known to possess characteristics of biological activity, activity is likely the result of a combination of factors including CT structural and compositional characteristics as well as CT concentration.

Based on *in vitro* results, North American native-warm season perennial legumes containing biologically active CT have promise for use in ruminant diets for the purpose

of suppressing enteric CH_4 emissions and subsequently reducing loss of gross energy intake in ruminants. In addition, they potentially provide rumen bypass protein, which could increase efficiency of protein utilization by ruminants and alter the form of N excreted by the animal. Further evaluation of the phytochemical characteristics of CT from the warm-season perennial legumes, relative to suppression of CH_4 emissions, protein binding ability and inhibition of L3 stage *Haemonchus contortus* larval migration is needed to identify possible contributing factors to CT biological activity.

The identification of key characteristics of forage legume CT that contribute to biological activity has important implications in plant-herbivore interactions and the ability to harness the potential of plant polyphenolics as a beneficial and sustainable resource to both wild and domestic ruminants. However, a more holistic rather than reductionist approach to evaluating these characteristics will likely one day lead to the identification of the multiple facets of CT that combine to confer biological activity to forage CT and benefit sustainable ruminant animal production.

LITERATURE CITED

Aerts, R.J., W.C. McNabb, A. Molan, A. Brand, T.N. Barry, and J.S. Peters. 1999. Condensed tannins from *Lotus corniculatus* and *Lotus pedunculatus* exert different effects on the *in vitro* rumen degradation of ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco) protein. J. Sci. Food Agric. 79:79-85.

Animut, G., R. Puchala, A.L. Goetsch, A.K. Patra, T. Sahlu, V.H. Varel, and J. Wells. 2008. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Anim. Feed Sci. Technol. 144:212–227.

Armstrong, S.A., D.R. Klein, T.R. Whitney, C.B. Scott, J.P. Muir, B.D. Lambert, and T.M. Craig. "In Press". Effect of using redberry juniper (*Juniperus pinchotii*) to reduce *Haemonchus contortus in vitro* viability and increase ivermectin efficacy. Vet. Parasitol.

Athanasiadou, S., I. Kyriazakis, F. Jackson, and R.L. Coop. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. Vet. Parasitol. 99:205–219.

Azuhnwi, B.N., B. Thomann, Y. Arrigo, B. Boller, H.D. Hess, M. Kreuzer, and F. Dohme-Meier. 2012. Ruminal dry matter and crude protein degradation kinetics of five sainfoin (*Onobrychis viciifolia* Scop) accessions differing in condensed tannin content and obtained from different harvests. Anim. Feed Sci. Tech. 177:135-143.

Bae, Y.S., L.Y. Foo, J.J. Karchesy. 1994. GPC of natural procyanidin oligomers and

polymers. Holzforschung 48:4-6.

Barry, T.N., and W.C. McNabb. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Brit. J. Nutr. 81:263–272.

Bate-Smith E.C. 1975. Phytochemistry of proanthocyanidins. Phytochemistry 14:1107-1113.

Bate-Smith E.C. 1973a. Haemanalysis of tannins: The concept of relative astringency. Phytochemistry 12:907–912.

Bate-Smith E.C. 1973b. Tannins of herbaceous leguminosae. Phytochemistry 12:1809–1812.

Beart J.E., T.H. Lilley, and E. Haslam. 1985. Plant polyphenols-secondary metabolism and chemical defense: Some observations. Phytochemistry 24:33–38.

Brunet, S., F. Jackson, and H. Hoste. 2008a. Effects of sainfoin (*Onobrychis viciifolia*) extract and monomers of condensed tannins on the association of abomasal nematode larvae with fundic explants. Int. J. Parasitol. 38:783-790.

Brunet, S., C.M.O. de Montellano, J.F.J. Torres-Acosta, C.A. Sandoval-Castro, A.J. Aguilar-Caballero, C. Capetillo-Leal, and H. Hoste. 2008b. Effect of the consumption of *Lysiloma latisiliquum* on the larval establishment of gastrointestinal nematodes in goats. Vet. Parasitol. 157:81–88.

Buzzini P., P. Arapitsas, M. Goretti, E. Branda, B. Turchetti, P. Pinelli, F. Ieri, and A. Romani. 2008. Antimicrobial and antiviral activity of hydrolysable tannins. Mini Rev. Med. Chem. 8:1179–1187.

Canon F., R. Ballivian, F. Chirot, R. Antoine, P. Sarni-Manchado, J, Lemoine and P.

Dugourd. 2011. Folding of a salivary intrinsically disordered protein upon binding to tannins. J. Am. Chem. Soc. 133:7847–7852.

Carter E.B., M.K. Theodorou, and P. Morris. 1999. Responses of *Lotus corniculatus* to environmental change. 2. Effect of elevated CO_2, temperature and drought on tissue digestion in relation to condensed tannin and carbohydrate accumulation. J. Sci. Food Agric. 79:1431-1440.

Cenci, F.B., H. Louvandini, C.M. McManus, A. Dell'Porto, D.M. Costa, S.C. Araújo, A.P. Minho, and A.L. Abdalla. 2007. Effects of condensed tannin from *Acacia mearnsii* on sheep infected naturally with gastrointestinal helminthes. Vet. Parasitol. 144:132–137.

Child, R.D., E.K. Byington, and H.H. Hansen. 1985. Goats in the mixed hardwoods of the southeastern United States. p. 149–158. *In* Baker, F.H., Jones, R.K. (eds.), Multispecies Grazing. Winrock International Institute for Agricultural Development, Morrilton, Ark (USA).

Cortes, J.E., B. Moreno, M.L. Pabon, P. Avila, M. Kreuzer, H.D. Hess, and J.E. Carulla. 2009. Effects of purified condensed tannins extracted from *Calliandra, Flemingia* and *Leucaena* on ruminal and postruminal degradation of soybean meal as estimated *in vitro*. Anim. Feed Sci. Tech. 151:194-204.

Da Silva, J.M.R., V. Cheynier, J.M. Souquet, M. Moutounet, J.C. Cabanis, and M. Bourzeix. 1991. Interaction of grape seed procyandins with various proteins in relation to wine fining. J. Sci. Food Agric. 57:111-125.

Delehanty, J., B.J. Johnson, T.E. Hickey, T. Pons and F.S. Ligler. 2007. Binding and

neutralization of lipopolysaccharides by plant proanthocyanidins. J. Nat. Prod. 70:1718-1724.

Dinaburg, A.G. 1942. The efficiency of the Baermann apparatus in the recovery of larvae of *Haemonchus contortus*. J. Parasitol. 28:433–440.

Dixon, R.A., C. Liu, and J.H. Jun. 2012. Metabolic engineering of anthocyanins and condensed tannins in plants. Curr. Opin. Biotechnol. 24:1-7.

Dschaak, C.M., C.M. Williams, M.S. Holt, J.S. Eun, A.J. Young, and B.R. Min. 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. J. Dairy Sci. 94:2508–2519.

Edelmann, A., and B. Lendl. 2002. Toward the optical tongue: Flow-through sensing of tannin-protein interactions based on FTIR spectroscopy. J. Am. Chem. Soc. 124: 14741-14747.

Faithfull, N.T. 1984. The *in vitro* digestibility of feedstuffs–a century of ferment. J. Sci Food Agric. 35:819–826.

Feeny, P. and H. Bostock. 1968. Seasonal changes in the tannin content of oak leaves. Phytochemistry 7:871–880.

Fischer, D.G., S.C. Hart, B.J. Rehill, R.L. Lindroth, P. Keim, and T.G. Whitham. 2006. Do high-tannin leaves require more roots? Oecologia 149:668–675.

Foo, L.Y., W. Jones, L. Porter, and V.M. Williams. 1982. Proanthocyanidin polymers of fodder legumes. Phytochemistry 21:933–935.

Foo, L.Y., and L.J. Porter. 1980. The phytochemistry of proanthocyanidin polymers.

Phytochemistry 19:1747–1754.

Foster, J.L., J.P. Muir, B.D. Lambert, and D. Pawelek. 2007. *In situ* and *in vitro* degradation of native Texas warm-season legumes and alfalfa in goats and steers fed a sorghum-sudan basal diet. Anim. Feed Sci. Tech. 133:228–239.

Goering, H.K., and P.J. Van Soest. 1970. Forage fiber analysis: Apparatus, reagents, procedures, and some applications. Pages 1-20 in Agric. Res. Serv. Agric. Handbook. No. 379. USDA, Washington, DC.

Goldstein, J.L., and T. Swain 1965. The inhibition of enzymes by tannins. Phytochemistry 4:185–192.

Gurbuz, Y., M. Kaplan, and D.R. Davies. 2008. Effects of condensed tannin content on digestibility and determination of nutritive value of some selected native legumes species. J. Anim. Vet. Adv. 7:854–862.

Hagerman, A.E. 2012. Fifty years of polyphenol-protein complexes. Recent Advances in Polyphenol Research 3:71-97.

Hagerman, A.E., M.E. Rice, and N.T. Ritchard. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin(16) (4 -> 8) catechin (procyanidin). J. Agric. Food Chem. 46:2590-2595.

Hagerman, A.E. 1988. Extraction of tannin from fresh and preserved leaves. J. Chem. Ecol. 14:453-461.

Hagerman, A.E. 1987. Radial diffusion method for determining tannin in plant extracts. J. Chem. Ecol. 13:437-449.

Hagerman, A.E., and L.G.Butler. 1981. The specificity of proanthocyanidin-protein

interactions. J. Biol. Chem. 256:4494–4497.

Hagerman, A.E., and L.G. Butler. 1978. Protein precipitation method for the quantitative determination of tannins. J. Agric. Food Chem. 26:809-812.

Hillis, W.E. and T. Swain. 1959. The phenolic constituents of *Prunus domestica*: The Analysis of tissues of the victoria plum tree. J. Sci. Food Agric. 10:135–144.

Holtzapple, M.T., R.R. Davison, M.K. Ross, S. Aldrett-Lee, M. Nagwani, C.M. Lee, C. Lee, S. Adelson, W. Kaar, D. Gaskin, H. Shirage, N.S. Chang, V.S. Chang, and M.E. Loescher. 1999. Biomass conversion to mixed alcohol fuels using the MixAlco process. Appl. Biochem. Biotechnol. 77-79:609–631.

Horne, J., J. Hayes, and H.T. Lawless. 2002. Turbidity as a measure of salivary protein reactions with astringent substances. Chem. Senses 27:653–659.

Huang, X.D., J.B. Liang, H.Y. Tan, R. Yahya, and Y.W. Ho. 2011. Effects of *Leucaena* condensed tannins of differing molecular weights on *in vitro* CH_4 production. Anim. Feed Sci. Technol.166-167:373–376.

Huang, X.D., J.B. Liang, H.Y. Tan, R. Yahya, B. Khamseekhiew, and Y.W. Ho. 2010. Molecular weight and protein binding affinity of *Leucaena* condensed tannins and their effects on *in vitro* fermentation parameters. Anim. Feed Sci. Technol. 159:81–87.

Iqbal, Z., M. Sarwar, A. Jabbar, S. Ahmed, M. Nisa, M.S. Sajid, M.N. Khan, K.A. Mufti, and M. Yaseen. 2007. Direct and indirect anthelmintic effects of condensed tannins in sheep. Vet. Parasitol. 144:125–131.

Jones, W. T., and J. L. Mangan. 1977. Complexes of the condensed tannins of sainfoin

(*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. J. Sci. Food Agric. 28:126–136.

Kahiya, C., S. Mukaratirwa, and S.M. Thamsborg. 2003. Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. Vet. Parasitol. 115:265–274.

Kariuki, I.W., and B.W. Norton. 2008. The digestion of dietary protein bound by condensed tannins in the gastro-intestinal tract of sheep. Anim. Feed Sci. Tech. 142:197–209.

Kawamoto, H., F. Nakatsubo, and K. Murakami. 1995. Quantitative determination of tannin and protein in the precipitates by high-performance liquid chromatography. Phytochemistry 40:1503-1505.

Kennedy, J.A. and A.W. Taylor. 2003. Analysis of proanthocyanidins by high-performance gel permeation chromatography. J. Chromatogr. 995:99-107.

Koleckar, V., K. Kubikova, Z. Rehakova, K. Kuca, D. Jun, L. Jahodar, and L. Opletal. 2008. Condensed and hydrolysable tannins as antioxidants influencing the health. Mini-Rev. Med. Chem. 8:436-447.

Kongvongxay, S., T.R. Preston, R.A. Leng, and D.N. Khang. 2011. Effect of a tannin-rich foliage (*Mimosa pigra*) on feed intake, digestibility, N retention and methane production in goats fed a basal diet of *Muntingia calabura*. Livest. Res. Rural Dev. 23. http://www.lrrd.org/lrrd23/3/sito23048.htm.

Kraus, T.E.C., R.A. Dahlgren, and R.J. Zasoski. 2003. Tannins in nutrient dynamics of

forest ecosystems - a review. Plant Soil 256:41–66.

Krueger, W. K., H. Gutierrez-Bañuelos, G. E. Carstens, B. R. Min, W. E. Pinchak, R. R. Gomez, R. C. Anderson, N. A. Krueger, and T. D. A. Forbes. 2010. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass and non-carcass traits in steers fed a high-grain diet. Anim. Feed Sci. Technol. 159:1–9.

Li, C., R. Leverence, J.D. Trombley, S. Xu, J. Yang, Y. Tian, J.D. Reed, and A.E. Hagerman. 2010. High molecular weight persimmon (*Diospyros kaki* L.) proanthocyanidin: A highly galloylated, A-linked tannin with an unusual flavonol terminal unit, myricetin. J. Agric. Food Chem. 58:9033-9042.

Madritch, M.D., L.M. Jordan, and R.L. Lindroth. 2007. Interactive effects of condensed tannin and cellulose additions on soil respiration. Can. J. Forest Res. 37:2063–2067.

Makkar, H.P.S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Res. 49:241–256.

Mané, C., N. Sommerer, T. Yalcin, V. Cheynier, R.B. Cole, and H. Fulcrand. 2007. Assessment of the molecular weight distribution of tannin fractions through MALDI-TOF MS analysis of protein-tannin complexes. Anal. Chem. 79:2239–2248.

Mansfield, J.L., P.S. Curtis, D.R. Zak, and K.S. Pregitzer. 1999. Genotypic variation for condensed tannin production in trembling aspen (*Populus tremuloides,*

Salicaceae) under elevated CO_2 and in high and low fertility soil. Am. J. Bot. 86:1154–1159.

Marie-Magdeleine, C., M. Mahieu, L. Philibert, P. Despois, and H. Archimède. 2010. Effect of cassava (*Manihot esculenta*) foliage on nutrition, parasite infection and growth of lambs. Small Ruminant Res. 93:10–18.

Martin, J.S., and M.M. Martin. 1982. Tannin assays in ecological studies: Lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. Oecologia 54:205–211.

Max, R.A., A.E. Kimambo, A.A. Kassuku, L.A. Mtenga, and P.J. Buttery. 2007. Effect of tanniniferous browse meal on nematode faecal egg counts and internal parasite burdens in sheep and goats. S. Afr. J. Anim. Sci. 37:97–106.

Max, R.A., D. Wakelin, J. Craigon, A.A. Kassuku, A.E. Kimambo, L.A. Mtenga, and P.J. Buttery. 2005. Effect of two commercial preparations of condensed tannins on the survival of gastrointestinal nematodes of mice and goats *in vitro*. S. Afr. J. Anim. Sci. 35:213–221.

McArthur, C., G. Sanson, and A.M. Beal. 1995. Salivary proline-rich proteins in mammals: roles in oral homeostasis and counteracting dietary tannin. J. Chem. Ecol. 21:663–691.

McDougall, E.I. 1948. The composition and output of a sheep's saliva. Biochem. J. 43:99–109.

McGaw, L., D. Van der Merwe, and J. Eloff. 2007. *In vitro* anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African

ethnoveterinary medicine. Vet. J. 173:366–372.

McGraw, R.L., F.W. Shockley, J.F. Thompson, and C.A. Roberts. 2004. Evaluation of native legume species for forage yield, quality, and seed production. Native Plants J. 5:152–159.

McSweeney, C.S., J. Gough, L.L. Conlan, M.P. Hegarty, B. Palmer, and D.O. Krause. 2005. Nutritive value assessment of the tropical shrub legume *Acacia angustissima*: Anti-nutritional compounds and *in vitro* digestibility. Anim. Feed Sci. Tech. 121:175–190.

Meagher, L.P., G. Lane, S. Sivakumaran, M.H. Tavendale, and K. Fraser. 2004. Characterization of condensed tannins from species by thiolytic degradation and electrospray mass spectrometry. Anim. Feed Sci. Tech. 117:151-163.

Meagher, L.P., K. Widdup, S. Sivakumaran, R. Lucas, and W. Rumball. 2006. Floral *Trifolium* proanthocyanidins: polyphenol formation and compositional diversity. J. Agric. Food Chem. 54:5482–5488.

Mehlich, A. 1984. Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. Comm. Soil Sci. Plant An. 15:1409-1416.

Min, B.R., W.E. Pinchak, J.D. Fulford, and R. Puchala. 2005. Wheat pasture bloat dynamics, *in vitro* ruminal gas production, and potential bloat mitigation with condensed tannins. J. Anim. Sci. 83:1322–1331.

Minho, A.P., I.C.S. Bueno, H. Louvandini, F. Jackson, S.M. Gennari, and A.L. Abdalla. 2008. Effect of *Acacia molissima* tannin extract on the control of gastrointestinal parasites in sheep. Anim. Feed Sci. Tech. 147:172–181.

Mole, S., L.G. Butler, and G. Iason. 1990. Defense against dietary tannin in herbivores: A survey for proline rich salivary proteins in mammals. Biochem. Syst. Ecol. 18:287–293.

Mole , S., and P.G. Waterman. 1987. A critical analysis of techniques for measuring tannins in ecological studies. Oecologia. 72:137-147.

Monagas, M., J.E. Quintanilla-López, C. Gómez-Cordovés, B. Bartolomé, and R. Lebrón-Aguilar. 2010. MALDI-TOF MS analysis of plant proanthocyanidins. J. Pharm. Biomed. Anal. 51:358–372.

Montellano, C.M.O., J.J. Vargas-Magaña, H.L. Canul-Ku, R. Miranda-Soberanis, C. Capetillo-Leal, C.A. Sandoval-Castro, H. Hoste, and J.F.J. Torres-Acosta. 2010. Effect of a tropical tannin-rich plant *Lysiloma latisiliquum* on adult populations of *Haemonchus contortus* in sheep. Vet. Parasitol. 172:283–290.

Muir, J.P. 2011. The multi-faceted role of condensed tannins in the goat ecosystem. Small Ruminant Res. 98:115–120.

Muir, J.P., W.D. Pitman, and J.L. Foster. 2011. Sustainable, low-input, warm-season, grass-legume grassland mixtures: mission (nearly) impossible? Grass Forage Sci. 66:301–315.

Muir, J.P., J. Taylor, and S.M. Interrante. 2005. Herbage and seed from Texan native perennial herbaceous legumes. Rangeland Ecol. Manag. 58:643–651.

Naumann, H.D., L.O. Tedeschi, J.P. Muir, B.D. Lambert, and M.M. Kothmann. 2013a. Effect of molecular weight of condensed tannins from warm-season perennial

legumes on ruminal methane production *in vitro*. Biochem. Syst. Ecol. 50:154-162

Naumann, H.D., J.P. Muir, B.D. Lambert, L.O. Tedeschi, and M.M. Kothmann. 2013b. Condensed tannins in the ruminant environment: A perspective on biological activity. J. Agric. Sci. 1:8-20.

Newbold, C.J., S. Lopez, N. Nelson, J.O. Ouda, R.J. Wallace, and A.R. Moss. 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*. Br. J. Nutr. 94:27-35.

Núñez, V., C. Gómez-Cordovés, B. Bartolomé, Y.J. Hong, and A.E. Mitchell. 2006. Non-galloylated and galloylated proanthocyanidin oligomers in grape seeds from *Vitus vinifera* L. cv. Graciano, Tempranillo and Cabernet Sauvignon. J. Sci. Food Agric. 86:915–921.

O'Donovan, L., and J.D. Brooker. 2001. Effect of hydrolysable and condensed tannins on growth, morphology and metabolism of *Streptococcus gallolyticus* (*S. caprinus*) and *Streptococcus bovis.* Microbiology. 147:1025-1033.

Okuda, T. and H. Ito. 2011. Tannins of constant structure in medicinal and food plants—Hydrolyzable tannins and polyphenols related to tannins. Molecules 16:2191–2217.

Patra, A.K., and J. Saxena. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91:24-37.

Patra, A.K., and J. Saxena. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71:1198–1222.

Peleg, H., K. Gacon, P. Schlich, and A.C. Noble. 1999. Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. J. Sci. Food Agric. 79:1123–1128.

Pellikaan, W.F., E. Stringano, J. Leenaars, D.J.G.M. Bongers, S.V.L.V. Schuppen, J. Plant, and I. Mueller-Harvey. 2011. Evaluating effects of tannins on extent and rate of *in vitro* gas and CH_4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166-167:377–390.

Perez-Maldonado, R.A., and B.W. Norton. 1996. The effects of condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* on protein and carbohydrate digestion in sheep and goats. Brit. J. Nutr. 76:515–533.

Pomroy, W. E., and B. A. Adlington. 2006. Efficacy of short-term feeding of sulla (*Hedysarum coronarium*) to young goats against a mixed burden of gastrointestinal nematodes. Vet. Parasitol. 136:363–366.

Prichard, R.K., 1990. Anthelmintic resistance in nematodes: Extent, recent understanding and future directions for control and research. Int. J. Parasitol. 20:515–523.

Puchala, R., G. Animut, A.K. Patra, G.D. Detweiler, J.E. Wells, V.H. Varel, and T. Sahlu. 2012. Effects of different fresh-cut forages and their hays on feed intake , digestibility , heat production , and ruminal methane emission by Boer × Spanish goats. J. Anim. Sci. 90:2754–2762.

Rahim, A.A., M.J. Kassim, E. Rocca, and J. Steinmetz. 2011. Mangrove (*Rhizophora apiculata*) tannins: an eco-friendly rust converter. Corros. Eng, Sci Technol 46:425–431.

Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73:1516-1528.

Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production *in vitro*. J. Dairy Sci. 81:3222–3230.

Salminen, J.P., M. Karonen, and J. Sinkkonen. 2011. Chemical ecology of tannins: Recent developments in tannin chemistry reveal new structures and structure-activity patterns. Chem. Eur. J. 17:2806-2816.

Sarni-Manchado, P., V. Cheynier, and M. Moutounet. 1999. Interactions of grape seed tannins with salivary proteins. J. Agric. Food Chem. 47:42-47.

Scioneaux, A.N., M.A. Schmidt, M.A. Moore, R.L. Lindroth, S.C. Wooley, and A.E. Hagerman. 2011. Qualitative variation in proanthocyanidin composition in *Populus* species and hybrides: Genetics is the key. J. Chem. Ecol. 37:57-70.

Shaik, S.A., T.H. Terrill, J.E. Miller, B. Kouakou, G. Kannan, R.M. Kaplan, J.M. Burke, and J.A. Mosjidis. 2006. Sericea lespedeza hay as a natural deworming agent against gastrointestinal nematode infection in goats. Vet. Parasitol. 139:150–157.

Shimada, T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32:1149–1163

Smith, A.H., E. Zoetendal, and R.I. Mackie. 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microbial Ecol. 50:197–205.

Soares, S., N. Mateus, and V. De Freitas. 2007. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. J. Agric. Food Chem. 55:6726-6735.

Spalinger, D.E., W.B. Collins, T.A.Hanley, N.E. Cassara, and A.M. Carnahan. 2010. The impact of tannins on protein, dry matter, and energy digestion in moose (*Alces alces*). Can. J. Zool. 88:977–987.

Stringano, E., A. Gea, J.P. Salminen, and I. Mueller-Harvey. 2011. Simple solution for a complex problem: Proanthocyanidins, galloyl glucoses and ellagitannins fit on a single calibration curve in high performance-gel permeation chromatography. J. Chromatogr. 1218:7804-7812.

Talbot, J.M., and A.C. Finzi. 2008. Differential effects of sugar maple, red oak, and hemlock tannins on carbon and nitrogen cycling in temperate forest soils. Oecologia 155:583–592.

Tan, H.Y., C.C. Sieo, N. Abdullah, J.B. Liang, X.D. Huang, and Y.W. Ho. 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and population of methanogens and protozoa *in vitro*.

Tavendale, M.H., L.P. Meagher, D. Pacheco, N. Walker, G.T. Attwood, and S. Sivakumaran. 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Technol. 123-124:403–419.

Tedeschi, L.O., T.R. Callaway, J.P. Muir, and R.C. Anderson. 2011. Potential environmental benefits of feed additives and other strategies for ruminant production. R. Bras. Zootec. 40:291–309.

Tedeschi, L.O., P.J. Kononoff, K. Karges, and M.L. Gibson. 2009. Effects of chemical composition variation on the dynamics of ruminal fermentation and biological value of corn milling (co) products. J. Dairy Sci. 92:401–413.

Terrill, T.H., G.S. Dykes, S.A. Shaik, J.E. Miller, B. Kouakou, G. Kannan, J.M. Burke, and J.A. Mosjidis. 2009. Efficacy of sericea lespedeza hay as a natural dewormer in goats: dose titration study. Vet. Parasitol. 163:52–56.

Terrill, T.H., A.M. Rowan, G.B. Douglas, and T.N. Barry. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agric. 58:321–329.

Terrill, T.H., W.R. Windham, J.J. Evans, and C.S. Hoveland. 1990. Condensed tannin concentration in sericea lespedeza as influenced by preservation method. Crop Sci. 30:219-224.

Tharayil, N., V. Suseela, D.J. Triebwasser, C.M. Preston, P.D. Gerard, and J.S. Dukes. 2011. Changes in the structural composition and reactivity of *Acer rubrum* leaf litter tannins exposed to warming and altered precipitation: climatic stress-induced tannins are more reactive. New Phytol. 191:132–145.

Tiemann, T.T., C.E. Lascano, H.R. Wettstein, A.C. Mayer, M. Kreuzer, and H.D. Hess. 2008. Effect of the tropical tannin-rich shrub legumes *Calliandra calothyrsus* and

Flemingia macrophylla on methane emission and nitrogen and energy balance in growing lambs. Animal 2:790-799.

Todd Jr, K.S., N. Levine, and P.A. Boatman. 1976. Effect of desiccation on the survival of infective *Haemonchus contortus* larvae under laboratory conditions. J. Parasitol. 62:247–249.

Venter, P.B., N.D. Senekal, G. Kemp, M. Amra-Jordaan, P. Khan, S.L. Bonnet, and J.H. Van der Westhuizen. 2012. Analysis of commercial proanthocyanidins. Part 3: The chemical composition of wattle (*Acacia mearnsii*) bark extract. Phytochemistry 83:153-167.

Verkaik, E., A.G. Jongkind, and F. Berendse. 2006. Short-term and long-term effects of tannins on nitrogen mineralisation and litter decomposition in kauri (*Agathis australis* (D. Don) Lindl.) forests. Plant Soil 287:337–345.

Veteli, T.O., W.J. Mattson, P. Niemelä, R. Julkunen-Tiitto, S. Kellomäki, K. Kuokkanen, and A. Lavola. 2007. Do elevated temperature and CO_2 generally have counteracting effects on phenolic phytochemistry of boreal trees? J. Chem Ecol. 33:287–296.

Vidal, S., L. Francis, S. Guyot, N. Marnet, M. Kwiatkowski, R. Gawel, V. Cheynier, and E.J. Waters. 2003. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. J. Sci. Food Agric. 83:564–573.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci. Technol. 147:116–139.

Whitley, N. C., J.E. Miller, J.M. Burke, D. Cazac, D.J. O'Brien, L. Dykes, and J.P. Muir. 2009. Effect of high tannin grain sorghum on gastrointestinal parasite fecal egg counts in goats. Small Ruminant Res. 87:105–107.

Whitney, T.R., A.E. Lee, D.R. Klein, C.B. Scott, and T.M. Craig. 2010. A modified *in vitro* larvae migration inhibition assay using rumen fluid to evaluate *Haemonchus contortus* viability. Vet. Parisitol. 176:217–225.

Whitney, T.R., A.E. Lee, D.R. Klein, C.B. Scott, T.M. Craig, and J.P. Muir. 2011. A modified *in vitro* larvae migration inhibition assay using rumen fluid to evaluate *Haemonchus contortus* viability. Vet. Parasitol. 176:217-225.

Williams, C.M., J.S. Eun, J.W. MacAdam, A.J. Young, V. Fellner, and B.R. Min. 2011. Effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms. Anim. Feed Sci. Technol. 166-167:364–372.

Williams, V.M., L.J. Porter, and R.W. Hemingway. 1983. Molecular weight profiles of proanthocyanicin polymers. Phytochemistry 22:569–572.

Wolfe, R.M., T.H. Terrill, and J.P. Muir. 2008. Drying method and origin of standard affect condensed tannin (CT) concentrations in perennial herbaceous legumes using simplified butanol-HCl CT analysis. J. Sci. Food Agric. 88:1060–1067.

Yan Q., and A. Bennick. 1995. Identification of histatins as tannin-binding proteins in human saliva. Biochem. J. 311: 341-347.

Yokota, K., H. Kimura, S. Ogawa, and T. Akihiro. 2013. Analysis of A-Type and B-Type highly polymeric proanthocyanidins and their biological activities as nutraceuticals. J. Chem.

Yoshida, T., T. Hatano, and H. Ito. 2005. High molecular weight plant polyphenols (tannins): Prospective functions. In: Romeo JT, (ed.). Chemical Ecology and Phytochemistry of Forest Ecosystems. San Diego, CA, USA: Elsevier Inc. p 163-190.